The Analysis of Transgenic Mice with Mutations Involving Cartilage Extracellular Matrix Proteins, Collagen Type II and Cartilage Oligomeric Matrix Protein

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TABLE OF CONTENTS

	Page
INDEX OF FIGURES	ii
INDEX OF TABLES	iv
ACKNOWLEDGEMENTS	v
ABSTRACT	vii
INTRODUCTION	1
Cartilage	1
Collagen Type II	19
Human Collagen Type II Mutations	26
Mouse Models with Collagen Type II Mutations	31
Strategies for therapeutic Intervention	34
Research Goals	41
MATERIALS AND METHODS	43
CHAPTER 1 A NON-GLYCINE TYPE II COLLAGEN MUTATION DISRUPTS SKELETAL DEVELOPMENT IN TRANSGENIC MICE	53
Background	54
Results	63
Discussion	83
CHAPTER 2 THE ANALYSIS OF TRANSGENIC MICE WITH AN ASSEMBLY MUTATION IN CARTILAGE OLIGOMERIC MATRIX PROTEIN	87
Background	88
Results	108
Discussion	129
SUMMARY AND CONCLUSIONS	134
REFERENCES	138
APPENDICES	
Molecular Modeling of Collagen Type II R789C Mutation	190
Mouse COMP coiled-coil domain sequence	196

INDEX OF FIGURES

		Page
Figure Intro 1	*Endochondral bone formation.*	4
Figure Intro 2	*Collagen fibrils.*	22
Figure Intro 3	*Collagen type II expression vector.*	35
Figure 1.1	*Spondyloepiphyseal Dysplasia patient.*	59
Figure 1.2	*Ribozyme cleavage experiment.*	61
Figure 1.3	*Genotype and phenotype of R789C mice.*	64
Figure 1.4	*Histological analysis of the growth plate.*	68
Figure 1.5	*Immunohistochemistry of growth plate cartilage.*	70
Figure 1.6	*Electron microscopy of cells and matrix from cartilage.*	72
Figure 1.7	*Immuno-electron microscopy of cartilage matrix.*	74
Figure 1.8	*Transgene expression.*	78
Figure 1.9	*Protein analysis of transfected rat chondrosarcoma cells.*	80
Figure 2.1	*COMP pentameric and monomeric structure.*	96
Figure 2.2	*PSACH and MED patients.*	100
Figure 2.3	*Truncated-COMP construct.*	109
Figure 2.4	*Genotype and phenotype of transgenic mice.*	111
Figure 2.5	*Histological analysis of growth plate cartilage.*	118
Figure 2.6	*Immunohistology of the growth plate cartilage.*	120
Figure 2.7	*EM of the cartilage cells and matrix from newborn mice.*	124
Figure 2.8	*Protein analysis of chondrocytes from transgenic mice.*	127

Figure A.1 *Molecular Modeling of R789C Mutation.* 192

Figure A.2 *Sequence analysis of mouse COMP coiled-coil domain.* 197

INDEX OF TABLES

		Page
Table 2.1	*Statistical analysis of skeletal measurements in female wild-type and transgenic mice.*	115
Table 2.2	*Statistical analysis of skeletal measurements in male wild-type and transgenic mice.*	115
Table A.1	*Non-bonded interaction energies involving R789, C789, and the surrounding residues.*	194

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ABSTRACT

Chondrodysplasias are inherited disorders of bone formation. Patients suffer from skeletal deformities, associated with or without dwarfism, in varying degrees. Although other tissues may be involved, cartilaginous tissues are primarily affected. Cartilage has many important roles in the formation, growth and maintenance of the skeleton. My thesis work has focused on analyzing the skeletal consequences of two transgenic mouse lines with mutations in cartilage extracellular matrix proteins, (1) type II collagen and (2) cartilage oligomeric matrix protein.

Spondyloepiphyseal Dysplasia congenita is a dwarfing condition involving a shortened trunk and extremities. It has previously been found to be associated with a recurrent mutation, R789C, in the COL2A1 gene. The altered amino acid is on the outside of the triple helix of the collagen molecule, in the Y position of the Gly-X-Y repeats, where changes are usually tolerated. To better understand the mechanism of this mutation and to develop a ribozyme based gene therapy to target the mutant transcript, a transgenic mouse model was generated. In contrast to the human phenotype, the F1 transgenic littermates were severely affected.

Affected mice featured short limbs, a shortened face and cleft palate, while dying soon after birth. Light microscopy revealed a disorganized growth plate, while electron microscopy showed an extreme deficiency of collagen fibrils in the transgenic mouse cartilage. Collagen molecules containing mutant α chains were retained in cells stably transfected with the transgene. In conclusion, cartilage, deficient of collagen type II, causes a severe skeletal phenotype in transgenic mice.

Secondly, cartilage oligomeric matrix protein (COMP) is a pentameric, non-collagenous glycoprotein. Each COMP pentamer is composed of five monomers, which are assembled by a coiled-coil domain. Mutations within the COMP gene cause two types of dominantly inherited chondrodysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED). These disorders are characterized by mild to severe short stature and early onset osteoarthritis. The effects of COMP mutations in these patients suggest that COMP function is essential for normal bone growth. However, there is a limited understanding of how COMP is involved in this process.

In an attempt to understand the function of COMP, transgenic mice were generated which were hypothesized to have a disruption in normal COMP assembly. By overexpressing a truncated monomer containing only the coiled-coil domain, COMP molecules were expected to have a combination of normal arms and shorter arms. The transgenic F1 offspring have normal skeletal development and are fertile. Histologically, the growth plate and cartilage matrix composition is normal. Statistical analysis in the adult mice (five months old) suggest that there is no correlation between the COMP genotype and size. Furthermore, there is no evidence of arthritis in the transgenic mice at 14 months old. Protein analysis did not confirm the presence of a transgenic product, only that normal COMP assembly was not disturbed in transgenic samples.

INTRODUCTION

Collagen type II is the primary structural component in cartilage. Assembled collagen fibrils interact with other cartilage extracellular matrix proteins to ensure proper development and growth of the vertebrate skeleton. Over 40 mutations in the gene encoding collagen type II (*COL2A1*) have been described in patients with a variety of skeletal disorders (Horton and Hecht, 2000; Kuivaniemi et al., 1997). These disorders range from a lethal dwarfing condition, such as achondrogenesis type II, to a patient with normal stature and early onset osteoarthritis. In an attempt to understand these disorders several transgenic mice have been generated which express mutated COL2A1 genes (Garofalo et al., 1991; Li et al., 1995; Maddox et al., 1997; Metsäranta et al., 1992; Vandenberg et al., 1991). These mice show phenotypes similar to some forms of human chondrodysplasias. Disorders of skeletal development, both in humans and mice, emphasize the importance of collagen type II in cartilage structure and function.

CARTILAGE

Cartilage is a specialized form of connective tissue found throughout most of the human body. It is primarily an avascular tissue being composed of only cartilage cells surrounded by a complex extracellular matrix. In adult tissue, cartilage is solid and firm though slightly flexible. Cartilage has a primary role in allowing the body to resist compressional forces, particularly in the joints. Cartilage also plays many important roles in the formation and growth of the vertebrate skeleton. A healthy skeleton depends upon three types of cartilage for its development, growth, function and maintenance: hyaline,

elastic and fibrocartilage. Each are generally categorized by appearance and extracellular matrix composition (Morris et al., 2000).

Hyaline cartilage is the most abundant type in the human body. Upon appearance, it is shiny and translucent. During bone development, hyaline cartilage serves as a structural template on which bone matrix is eventually laid. Hyaline cartilage is present within the growth plate of developing bones until maturation is attained. Hyaline cartilage is also found at joint surfaces where it is present throughout life. The chondrocytes are generally oval in shape, but tend to be round towards the center of the tissue and quite flat nearing the periphery (Stockwell, 1979). Hyaline chondrocytes produce a complex array of extracellular matrix proteins designed to distribute the weight load on a particular bone. The matrix is composed of collagen fibrils which form a meshwork with proteoglycans and other matrix proteins. This specialized matrix allows hyaline cartilage to resist strong compressional forces, such as on articular surfaces.

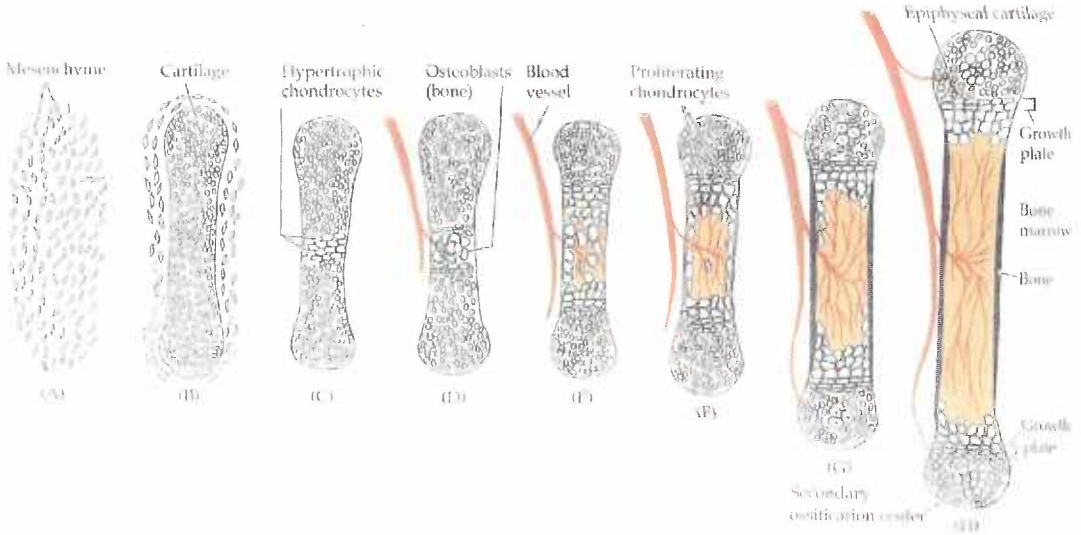
Fibrocartilage is often described as having qualities of an intermediate tissue with features of both hyaline cartilage and dense fibrous tissue (Stockwell, 1979). It is typically found in the intervertebral discs, the symphysis pubis and the menisci of the knee joints. As it merges with nearby structures, it begins to morphologically resemble that particular tissue whether it is cartilage or tendon. Furthermore, it has the ability to withstand compressive forces, as hyaline cartilage does, and to resist shear forces, as tendon does. The chondrocytes are typically like other cartilage cells having a round to oval shape. Cartilage matrix constituents vary depending on tissue requirements needed at that anatomical location.

Finally, elastic cartilage is similar to hyaline cartilage. It is located in the larynx, auditory canal and auditory tube. The chondrocyte shape is round to oval with the cells towards the edge of the tissue being quite flat (Anderson, 1964). However, the extracellular matrix is quite different from that of hyaline cartilage. The matrix is dense with an elastin network while the collagen fibrils are, in contrast, very thin. The elastin network, produced by chondrocytes, allows the tissue to be flexible and to withstand stretch, such as that found in the external ear.

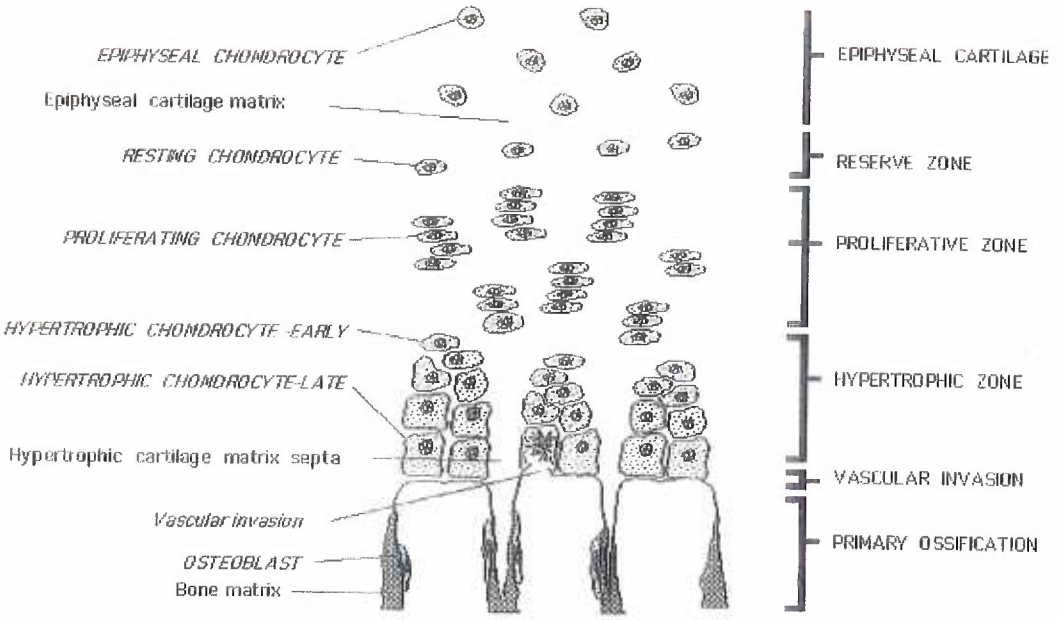
Although each type of cartilage is well adapted to serve in various connective tissue roles, hyaline cartilage performs very important roles in skeletal development and growth. For the majority of the skeleton, hyaline cartilage provides a template for future bones to be built. As cartilage becomes bone, chondrocytes progress through a series of stages with each stage requiring the secretion of specific extracellular matrix proteins and growth factors. Through the coordination of chondrogenesis and osteogenesis, bone matrix eventually replaces cartilage. This process is called endochondral ossification (Gilbert, 1997; Horton, 1990) (Figure Intro-1A).

Endochondral Ossification

Bone formation is an extremely complex, tightly regulated process that begins in embryonic development. Bone development can occur by either intramembraneous ossification or by endochondral ossification. Intramembraneous ossification is a process that converts mesenchymal cells directly into bone. The flat bones of the skull develop in this manner. Endochondral ossification also begins with mesenchymal cells; however, these cells differentiate into chondrocytes before becoming bone (Figure Intro-1A).


Mesenchyme
Cartilage
Hypertrophic chondrocytes
Osteoblasts (bone)
Blood vessel
Proliferating chondrocytes
Epiphyseal cartilage
Growth plate
Bone marrow
Bone
Growth plate
Secondary ossification center

A


EPIPHYSEAL CHONDROCYTE
Epiphyseal cartilage matrix
RESTING CHONDROCYTE
PROLIFERATING CHONDROCYTE
HYPERTROPHIC CHONDROCYTE-EARLY
HYPERTROPHIC CHONDROCYTE-LATE
Hypertrophic cartilage matrix septa
Vascular invasion
OSTEOBLAST
Bone matrix
EPIPHYSEAL CARTILAGE
RESERVE ZONE
PROLIFERATIVE ZONE
HYPERTROPHIC ZONE
VASCULAR INVASION
PRIMARY OSSIFICATION

B

Most of the skeleton develops from endochondral ossification, including the vertebral column, base of the skull, shoulder girdle, pelvis, and limbs.

Chondrocytes produce the cartilage matrix which becomes a template for bone matrix to be deposited on. Cartilage formation can be divided into three phases: (1) mesenchyme proliferation, (2) mesenchyme condensation and (3) chondrocyte differentiation. Mesenchyme is initially composed of uniform, sparsely packed cells. As the cells multiply, the patterned expression of homeobox genes conveys information, like bone shape and position, to the mesenchymal cells (Eriebacher et al., 1995). These cells proliferate and begin to condense in areas predetermined to become bone (Hall and Miyake, 1995). At this stage, the cells secrete collagen types I, IIA (an alternatively spliced form) and III, fibronectin and very small quantities of noncartilaginous proteoglycans (Dessau et al., 1980; Frenz et al., 1989; Lui et al., 1995; Sandell et al., 1991; Sandell et al., 1994; Silver et al., 1981; Solursh, 1984).

There are several secreted molecules which are important for mesenchymal condensation and chondrocyte differentiation. The bone morphogenetic proteins (BMPs) are a family of secreted signaling molecules that belong to the transforming growth factor beta (TGF-β) superfamily. BMPs have been shown to induce ectopic cartilage formation in tissues, such as in muscle or connective tissues, and are thought to play many different roles in skeletogenesis (Hötten et al., 1996). GDF5, a BMP family member, has been reported to enhance the commitment of mesenchymal cells into the chondrocyte lineage (Tsumaki et al., 1999). In addition, neural cell adhesion molecules (NCAM) and N-cadherin are cell surface adhesion molecules, which have been reported to have roles in the formation and maintenance of precartilaginous condensations (Hall and Miyake,

1995; Oberlender and Tuan, 1994; Widelitz et al., 1993). The mechanisms by which these molecules act are not completely understood.

After the precartilaginous mesenchymal cells condense, they begin expressing cartilage specific proteins, such as collagen type II and aggrecan (Kosher et al., 1986; Nah et al., 1988). These cells, now referred to as chondrocytes, will continue to produce cartilage matrix until the bone template is formed (Figure Intro-1A). Surrounding each "cartilage" condensation, a thin layer of peripheral cells differentiate into the perichondrium. Through the expression of *bone morphogenetic protein 7* (*BMP-7*) and *parathyroid hormone/parathyroid hormone-related peptide receptor* (*PTH/PTHrP receptor*), the perichondrium controls chondrocyte proliferation and maturation (Long and Lisenmayer, 1998; Vortkamp et al., 1996).

After the formation of cartilage models is complete, cartilage cells proceed through a series of differentiation steps to ultimately be replaced by bone. Chondrocytes exhibit phenotypes of four stages: resting, proliferating, prehypertrophic or hypertrophic (Figure Intro-1B). At the epiphyseal end of the bone lie the resting, immature chondrocytes awaiting the signal to proliferate. Moving towards the center of the bone, the proliferating chondrocytes are found. Adjacent to the proliferative zone are the larger and more sparsely distributed prehypertrophic chondrocytes. These cells produce *Indian Hedgehog* (*Ihh*). Through *Ihh* and *BMP* signaling, which eventually results in the periarticular expression of *PTHrP* and activation of PTH/PTHrP receptor, prehypertrophic chondrocytes can negatively control the number of cells entering the prehypertrophic stage (Lanske et al., 1996; Vortkamp et al., 1996; Zou et al., 1997).

As the hypertrophic chondrocytes terminally differentiate and undergo apoptosis, bone matrix begins to be deposited on remnants of degraded cartilage (Horton, 1990). The *Delta-1/Notch* signaling pathway has been shown to influence chondrocyte maturation to the hypertrophic stage (Crowe et al., 1999). Hypertrophic chondrocytes produce a different matrix, containing collagens, particularly types I and X, fibronectin and less protease inhibitors (Dessau et al., 1978; Horton and Machado, 1988; Schmid et al., 1990). As the cartilage matrix is degraded, the hypertrophic cells undergo apoptosis. The secretion of vascular endothelial growth factor (VEGF) promotes invasion of bone marrow capillaries and iniatiates cell death (Gerber et al., 1999). Osteoblasts, entering through blood vessels, attach to residual matrix septa and hypertrophic cartilage and begin to secrete bone matrix.

As bone matrix replaces cartilage, an ossification front is created between the newly synthesized bone and the remaining cartilage (Figure Intro-1A). This front moves from the center of the cartilage template towards the ends of the bone replacing the recently differentiated hypertrophic chondrocytes with bone cells and matrix. However, when the front approaches the end of the bone, chondrocytes proliferate prior to undergoing hypertrophy. This newly formed cartilaginous region is called the epiphyseal growth plate. Growth plates are located at each end of a long bone maintaining growth throughout development (Figure Intro-1A, B). Hyaline cartilage, at the epiphyseal growth plate, is under constant change and is not permanent. After the bone has formed into the correct shape and grown to its desired length, epiphyseal cartilage is then transformed into bone. The growth plate is now closed. Thereafter, adult bones are continuously remodeled throughout life.

Endochondral bone growth is a highly regulated process that requires the presence and cooperation of many proteins. Signaling molecules, such as growth factors and hormones, are needed to govern the transformation from cartilage to bone. Through *in vitro* and *in vivo* transgenic and gene targeting experiments, many of the signaling molecules have been identified and their importance in skeletal development has been determined. The biological roles of most of these proteins continue to be investigated. However, bone growth also requires a complex extracellular matrix in which to transport, store and to deliver these signals. The extracellular matrix is a dynamic structure that involves interactions between matrix molecules or with cell surface receptors to initiate signaling pathways. To understand cartilage structure and function, it is necessary to discuss the proteins that make up its extracellular matrix.

Extracellular Matrix of Hyaline Cartilage

Cartilage is a unique tissue due to its high extracellular matrix (ECM) content and its relatively low cell density. The matrix is composed of a variety of proteins and polysaccharides that are secreted locally and assembled into an organized meshwork. In addition to binding chondrocytes together, the ECM also influences their development, polarity and behavior. The ECM makes up approximately 90% of adult articular cartilage and 50% of fetal cartilage and is primarily composed of collagens and proteoglycans (Heinegård and Oldberg, 1989).

Collagens are the most abundant components of the ECM and provide an fibrous matrix entangling other matrix proteins. Collagen fibrils are highly organized polymers of triple helical collagen molecules which can assemble into a variety of ordered arrays.

Fibrils are thin structures approximately 10-300 nm in diameter and many hundreds of micrometers long in mature tissues. Collagen fibrils often aggregate into larger, cablelike bundles called collagen fibers. Cartilage collagen fibrils form the basic meshwork which keeps large proteoglycans in place and are essential for maintaining the resilient properties of cartilage.

Proteoglycans and glycosaminoglycans (GAGs) associate to form huge polymeric complexes in the ECM. Proteoglycans are composed of GAG chains that are linked to a core protein. GAGs are unbranched polysaccharide chains composed of repeating disaccharide units. Proteoglycan complexes are completely variable in the size of core protein and the number or type of GAG chains that are associated. The heterogeneity of these GAGs makes it difficult to identify and classify proteoglycans by sugar content. Besides associating with one another, proteoglycans and GAGs associate with fibrous collagen matrices creating complex structures.

The cartilage ECM is extremely hydrated. Approximately 60-78% of the net weight of hyaline cartilage is water (Heinegård and Oldberg, 1989). Most of the water is bound to matrix proteins. Aggrecan, a chondroitin sulfate proteoglycan, is very abundant in cartilage and plays a major role in maintaining structural integrity. Due to the number of chondroitin and keratan GAG chains, aggrecan is capable of binding many water molecules. As the collagen fibrils constrain these aggregated proteins, an internal hydrostatic pressure is generated. This pressure enables cartilage to resist compressive forces on weight bearing joints. The presence of aggrecan is crucial for the maintenance of cartilage, for its absence is a major feature of arthritis (Dahlberg et al., 1994; Lohmander et al., 1993).

There are many other cartilage matrix proteins, not related to the collagens or to the proteoglycans, that are present in small amounts. Little is understood about the structural roles these minor proteins may play, but more recent transgenic work has shown their importance and they are being characterized further. I have organized this section by the functional units of the cartilage extracellular matrix: (1) heterotypic collagen fibrils, (2) specialized collagens, (3) aggregating proteoglycans, (4) non-aggregating proteoglycans and (5) other noncollagenous matrix proteins.

Heterotypic Collagen Fibrils

Collagens fibrils are the main structural proteins in the ECM of most tissues, including cartilage and bone. They play important roles in determining the size, shape and strength of these tissues. In mammals, at least 19 distinct collagen molecule types have been identified, along with a number of collagen-like molecules (Prockop and Kivirikko, 1995; Seyer and Kang, 1996). The collagens are a complex family that share similar triple-helical domains, composed of three α chains. The α chains may combine as homotrimers or as heterotrimers. Currently, at least 34 different α chains have been reported (Aumailley and Gayraud, 1998; Brown and Timpl, 1995; Cremer et al., 1998; van der Rest and Garrone, 1992). Roman numerals are used to identify the collagen type, while Arabic numerals are used to specify the individual polypeptide chains involved. For example, the alpha-1 chain of collagen type II is designated as α1(II) (Cole, 1994).

The polypeptide chains of collagen are arranged in a stretched polyproline II-like helix (Fraser et al., 1979; Rich and Crick, 1961; Rich and Crick, 1955; Ramachandran and Kartha, 1954). Each α chain has a Gly-X-Y sequence, where generally X is proline

and Y is hydroxyproline. Triple helix formation is dependent on the presence of a glycine, the smallest amino acid, in every third position. This allows the α chains to twist around one another along a central axis to create a right-handed superhelix (Brodsky and Shah, 1995). The X and Y residue side chains are located on the outside of the helix. The high content of the imino acids proline and hydroxyproline is important for the stability of the helix (Holmgren et al., 1998; Kühn, 1987). This structure is very resistant to proteolytic degradation, with the exception of specialized matrix metalloproteinases (Linsenmayer, 1991).

Cartilage collagen fibrils are made up primarily of type II, but also include types IX and XI (Cremer et al., 1998; Eyre and Wu, 1995; Mendler et al., 1989) (Figure Intro-2). Collagen type II is a member of the fibrillar collagen family with a 300nm triple helical domain and contributes 80-90% of the collagenous content of the fibril. It is cartilage specific in adult tissues; however, in embryogenesis, it can be found in noncartilaginous tissues, including notochord, heart, epidermis and brain (Cheah et al., 1991). Collagen type II is discussed in more detail later in this chapter.

Type IX is a fibril-associated collagen composed of three different chains (van der Rest and Mayne, 1987). It is found on the surface of type II collagen fibrils (van der Rest and Mayne, 1988; van der Rest et al., 1985). Crosslinks join the α-chains of type IX with the telopeptide termini of type II and other type IX molecules (Diab et al., 1996). There are several proposed functions of collagen type IX, which include a spacer between individual fibrils, a "glue" to bind the type II lattice and a means for collagen fibrils to interact with proteoglycan macromolecules (Douglas et al., 1998). Mice lacking the alpha 1 chain of type IX collagen have no detectable abnormalities at birth, but later

develop a severe degenerative joint disease similar to human osteoarthritis (Fässler et al., 1994). In the absence of this alpha chain, no collagen type IX is produced (Hagg et al., 1997). These results suggest that whatever the mechanism type IX collagen is important for maintaining the mechanical stability of articular cartilage.

Type XI collagen is also a member of the fibrillar collagen family and is composed of three different chains (Morris and Bächinger, 1987). Type XI is primarily located within cartilage collagen fibrils and covalently linked to itself (Petit et al., 1993; Wu and Eyre, 1995). It is thought that collagen type XI regulates fibrillar diameter, for it is more abundant in fibrils with smaller diameters (Keene et al., 1995). This idea was confirmed by the analysis of the chondrodysplasia (*cho*) mouse mutation where a single nucleotide deletion causes a frameshift and premature termination of the α1(XI) chain. Mice that are homozygous for the *cho* mutation are not viable (Li et al., 1995). These mice feature a severe phenotype with abnormalities in cartilaginous tissues including limbs, ribs, mandible and trachea. The mutant cartilage lacks type XI collagen and contains abnormally thick collagen fibrils with much greater interfibrillar space (Monson and Seegmiller, 1981; Seegmiller et al., 1988). These studies demonstrate that the presence of type XI collagen is essential for the formation of cartilage collagen fibrils and the regulation of collagen fibril size.

Specialized Collagens

Type X is a short-chain collagen and was one of the first cartilage matrix proteins to show a restricted distribution in cartilage. Type X collagen is specifically located in the hypertrophic zone of the developing growth plate cartilage at the interface with

endochondral ossification (Schmid et al., 1990) (Figure Intro-1B). It is also found in adult cartilage separating the articular cartilage from subchondral bone (Schmid and Linsenmayer, 1987). Type X collagen is a homotrimer and consists of three different regions. It has a short helical region, about half the size of the fibrillar collagens. A short non-helical region is located at the amino-terminus and a globular domain at the carboxyl-terminus. Within hypertrophic cartilage, type X is located pericellularly surrounding the chondrocytes and also associated with type II fibrils (Poole and Pidoux, 1989; Schmid et al., 1990).

Originally, mice lacking collagen type X were thought to have normal bone growth and development (Rosati et al., 1994). However, more recently collagen type X deficient mice are reported to have abnormal bone architecture, with reduced thickness of resting and articular cartilage and altered matrix distribution within the growth plate (Kwan et al., 1997). Dominant acting mutations found in humans and mice have bone abnormalities, resulting in Schmid metaphyseal chondrodysplasia (McIntosh et al., 1994; Warman et al., 1993). The function of collagen type X is not clear. It has been suggested that type X collagen plays a role in the distribution of matrix vesicles and proteoglycans in the growth plate.

Small amounts of types VI and XII are also present in cartilage (von der Mark et al., 1984). Type VI is a beaded filamentous collagen (Timpl and Engel, 1987). Homotrimeric and heterotrimeric forms of type VI occur containing shorter helical domains than type X and a much larger globular domain (von der Mark et al., 1984). Type XII is a fibril-associated collagen. It is a homotrimer and shares significant structural homology to type IX (Gordon et al., 1989). Collagens types VI and XII are not

specific to cartilage. They are also found in many other tissues, closely associated with collagen type I (von der Mark, 1984; Gorden et al., 1989; Cremer et al., 1998)

Aggregating Proteoglycans

Proteoglycans (PGs) are a diverse group of proteins that serve as attachment sites for one or more GAG chains (Doege et al., 1990). Aggrecan has a very large core protein, about 210 kDa, and is divided into several domains. In the mature PG, aggrecan has three globular domains and intervening regions which are bound to oligosaccharides and GAG chains (Doege et al., 1987). The large chondroitin sulfate domain, between G2 and G3, provides attachment for hundreds of GAG chains. Aggrecan binds hyaluronic acid (HA), the simplest GAG structure, at one site in the G1 domain. In addition, an amino-terminal domain interacts specifically with link protein (LP), which also contains a HA binding site (Wight et al., 1991). The ternary complex between aggrecan, HA and LP is very stable.

Aggrecan is a critical component for normal cartilage development because the ternary complex provides most of the water binding properties of the extracellular matrix. Cartilage matrix deficiency (cmd) mice were reported to have a small deletion in the aggrecan gene, resulting in a truncated molecule (Watanabe et al., 1994). The cmd mice have disproportionate dwarfism, a short snout, a cleft palate and died at birth. There is a severe reduction in the amount of aggrecan in the cartilage matrix, yet collagen type II and link protein were present at normal levels. Furthermore, nanomelia, a lethal chicken disease, is the result of a point mutation at the 5' end of the G3 domain of aggrecan. This mutation creates a premature stop codon, which prevents aggrecan from processing

properly (Argraves et al., 1981; Li et al., 1993; Vertel et al., 1993). These "loss of function" mutations strongly emphasizes the importance of aggrecan in cartilage development and in maintaining its structural integrity (Wai et al., 1998). No human mutations have been identified.

HA is a very large macromolecule and consists of a regularly repeating sequence of nonsulfated disaccharide units. A single molecule may have a molecular weight approaching 10 million (~25,000 repeat disaccharides) (Wight et al, 1991). Their open, random coil structures expand with water to form a viscous gel with varying pore sizes and charge densities which can affect cell-cell and cell-matrix interactions (Hall et al., 1995). HA is produced in large quantities during wound repair and it is a constituent of joint fluid, where it serves as a lubricant. Several molecules can bind HA through specific, noncovalent interactions.

LP is a glycoprotein that binds to aggrecan and HA and helps to stabilize the aggrecan complex. LP has great structural similarity to the G1 domain of aggrecan and are both able to bind to HA. Mice lacking LP showed severe delay in cartilage development and bone formation (Watanabe and Yamada, 1999). Null mice had short limbs and craniofacial abnormalities. Most died soon after birth due to respiratory problems; however, some survived and developed progressive dwarfism and lordosis of the cervical spine. The cartilage contained reduced aggrecan complexes in the hypertrophic zone, with a decrease in numbers of prehypertrophic and hypertrophic cells. The authors suggested that LP has a necessary role in proteoglycan aggregation and in the organization of hypertrophic chondrocytes.

Non-aggregating proteoglycans

Perlecan is a large multi-domain, heparan sulfate proteoglycan originally identified in basement membrane. Perlecan has been found to be essential for cartilage development. The gene encoding perlecan (*Hspg2*) has been disrupted in mice (Arikawa-Hirasawa et al., 1999). Some of the null mice (40%) died at embryonic stage 10.5 due to defective cephalic development. The remaining homozygous mice were lethal at birth and showed a severe skeletal dysplasia. Mice lacking perlecan have broad and bowed long bones, a narrow thorax and craniofacial abnormalities. Their cartilage features disorganized chondrocyte zones, reduced cartilage matrix and defective endochondral ossification. The authors have suggested that perlecan has a role in matrix structure.

Leucine-rich proteoglycans, such as decorin, biglycan, fibromodulin, lumican and epiphycan, are also components of cartilage (Hocking et al., 1998; Iozzo, 1998; Young et al., 1992). All of these molecules share similar sequence motifs. However, some of these proteins are only proteoglycans part of the time. Fibromodulin and lumican can also be found as glycoproteins with N-linked oligosaccharides that have not yet been modified to keratan sulfate chains (Grover et al., 1995; Roughley et al., 1996). In older human cartilage, biglycan is often present without its amino-terminal GAG-containing domain (Johnstone et al., 1993; Roughley et al., 1993). In contrast, decorin is primarily found as a proteoglycan at all tissue ages. As proteoglycans, these proteins are thought to have functional roles in collagen fibrillogenesis; however, as glycoproteins their roles are not clear (Hedbom and Heinegård, 1989; Pogány et al., 1994; Schönherr et al., 1995; Vogel et al., 1984).

Other Noncollagenous Matrix Proteins

Cartilage Oligomeric Matrix Protein (COMP) is a pentameric glycoprotein found in cartilage, ligament, tendon and synovium (DiCesare et al., 1994; Fife and Brandt, 1984; Hecht et al., 1998; Hummel et al., 1998; Maddox et al., 1997). Mutations within COMP have been found to cause two short-limbed dwarfing conditions, pseudoachondroplasia and multiple epiphyseal dysplasia (Briggs et al., 1995; Hecht et al., 1995). These patients often develop early osteoarthritis. Because of the effect on bone development and cartilage degradation, COMP is thought to have a crucial role in these processes. However, its function is still unclear (see chapter 2 for further discussion).

Cartilage Matrix Protein (CMP) or Matrillin I is a trimeric molecule with each chain connected by a carboxyl-terminal coiled-coil domain (Haudenschild et al., 1995). CMP expression is observed in early skeletal development. Later in life, CMP is only found in tracheal, nasal and auricular cartilages, not in articular cartilage or intervertebral discs (Aszodi et al., 1996; Mundlos and Zabel, 1994). CMP can bind to collagen type II and aggrecan or can self-associate (Chen et al., 1995; Winterbottom et al., 1992). It has been suggested that CMP may have a role in the organization and assembly of cartilage matrix (Winterbottom et al., 1992).

Several other cartilage matrix proteins have been identified, but their biological roles in hyaline cartilage are not understood. Chondrocalcin, the c-propeptide of procollagen type II, is abundant in fetal cartilage (Choi et al., 1983). It has an affinity for calcium and is located primarily where the growth plate is beginning to calcify (Poole et al., 1984). Chondrocalcin has been shown to bind the enhancer region of collagen type II gene, and may perhaps have a regulatory role in its expression (Nakata et al., 1996).

PARP, the n-propeptide of collagen α2 type XI, is abundant in epiphyseal cartilage and less abundant in adult articular cartilage (Neame et al., 1990). Chondroadherin (CHAD) is a leucine-rich protein that has been shown to promote chondrocyte attachment, possibly through integrin, α2β1 (Camper et al., 1997; Neame et al., 1994; Sommarin et al., 1989). CHAD is found in the growth plate and in articular cartilage. As the epiphysis develops, expression continues primarily in the proliferative zone (Shen et al., 1998).

In summary, hyaline cartilage is an extremely complex and specialized connective tissue which has specific roles in development and growth. The cartilage ECM is composed of an elaborate network of collagen fibrils, aggregating proteoglycans and several other minor proteins which are essential for cartilage function. The ECM is designed to provide structural support to the tissue through the interactions of other matrix molecules and the binding and release of water molecules. Because cartilage collagen fibrils form the underlying base for this complex matrix, it is not surprising that type II collagen, the primary constituent of the fibril, is a focal point for mutations which cause heritable skeletal dysplasias.

COLLAGEN TYPE II

Type II collagen is a homotrimer, composed of three identical α chains, α1(II) (Figure Intro-2A). It has a large helical domain, with 1014 amino acid residues per chain, which is flanked by two globular ends (propeptides). It's gene shares homology with the other major fibrillar collagens, types I and III. The human COL2A1 gene is approximately 30kb in size, containing 54 exons (Cole, 1994; Sandell and Boyd, 1990).

It is located on chromosome band 12q13.11-q13.12 (Takahashi et al., 1990). The N-propeptide is encoded by the first 7 exons. Exon 1 contains the untranslated 5' sequence and the signal peptide. The triple helical domain is encoded by exons 7-48. Like other fibrillar collagens, the exons encoding this domain are generally 54bp, in multiples of 54 or in combinations of 45 and 54bp. All exons, begin with a glycine residue and encode complete Gly-X-Y repeats (Ala-Kokko and Prockop, 1990). The C-propeptide is encoded by the next four exons, 49-52. Unlike other fibrillar collagens, type II collagen has two additional exons, 1B and 3B, which code for the amino-terminal domain (Chu and Prockop, 1993).

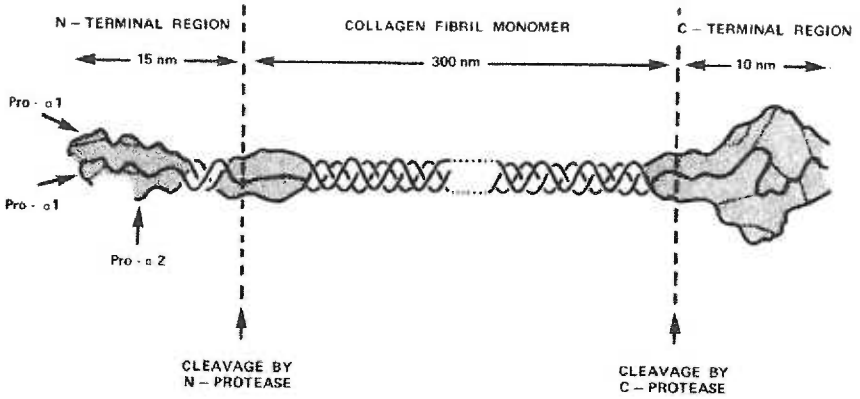
There are two alternatively spliced forms of type II collagen, type IIA and IIB (Metsäranta et al., 1991; Ryan et al., 1990). Exon 2 encodes a cysteine-rich region that is alternately spliced (Ryan and Sandell, 1990). Type IIA includes this exon, while type IIB does not (Sandell et al., 1991). Aside from the presence or absence of exon 2, the isoforms are identical. Type IIB collagen is expressed primarily by chondrocytes (Ryan and Sandell, 1990). It is the most abundant collagen in cartilage and, therefore, provides most of the structural support to this tissue. Type IIA has been detected throughout embryogenesis, in cartilage, notochord, heart, epidermis and brain (Cheah et al., 1991; Sandell et al., 1994). Collagen type IIA has been reported to bind to TGF-β1 and BMP-2 and may aid in distributing these growth factors through out cartilaginous tissues (Zhu et al., 1999). Throughout my thesis, "collagen type II" will refer to type IIB, unless stated otherwise.

Assembly of collagen molecules

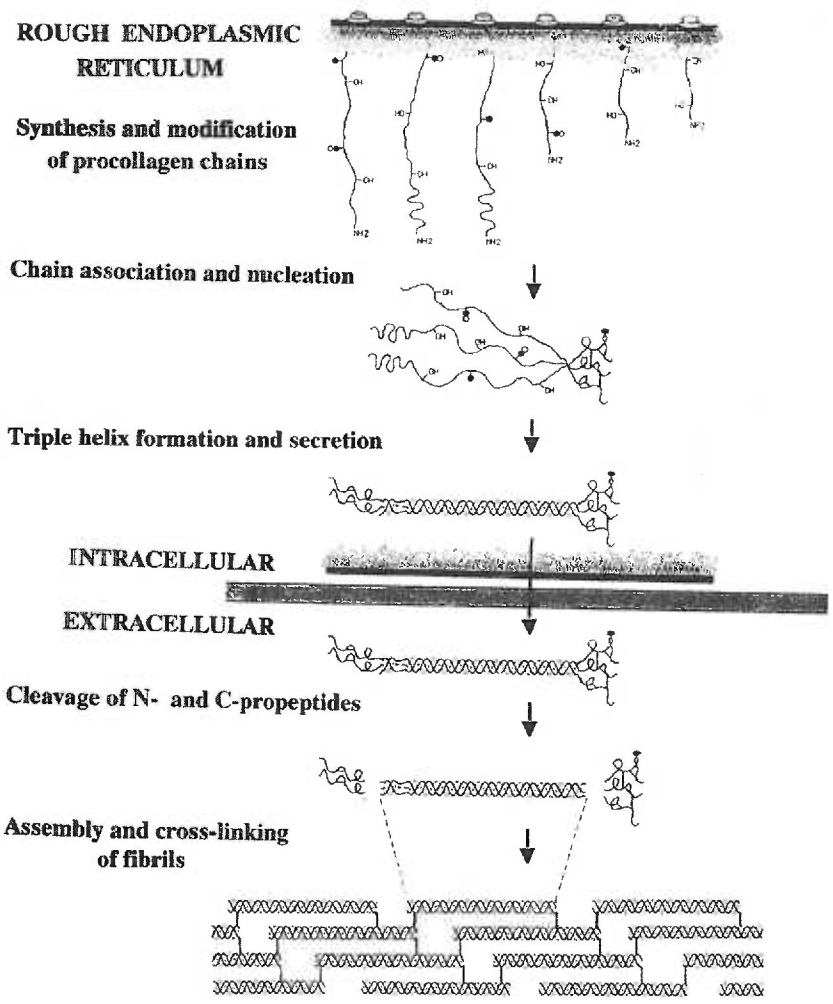
Collagen type II is first synthesized as a precursor procollagen molecule. The three α chains are translated and assembled into triple helical structures before exiting the cell. Procollagen molecules are made up of three distinct domains; a central triple helical domain and the N and C propeptide domains (Figure Intro-2A). In addition, telopeptide regions are located in between the helical domain and each end (not shown). Upon secretion to the extracellular matrix, proteolytic cleavage of the propeptides and assembly of the triple helical molecules into cross-banded fibrils occurs.

Single collagen polypeptide chains are synthesized on membrane-bound ribosomes and transported to the endoplasmic reticulum (ER) as a pro-α chain. These precursors have a short amino-terminal signal peptide, to direct the nascent chain to the ER lumen, and propeptides located at each end. Heat Shock Protein (HSP) 47 has been reported to bind the procollagen chain co-translationally and is thought to be involved in its translation and translocation to the ER (Satoh et al., 1996; Sauk et al., 1994). As the polypeptide chain is translocated across the ER membrane, it undergoes a series of modifications to specific amino acid side chains.

Several of these modifications occur within the triple helical repeats (Kivirikko et al., 1992). The hydroxylation of Y-position proline residues to 4-hydroxyproline provides added stability to the triple helix, however the mechanism is controversial (Berg et al., 1973; Germann and Heidemann, 1988; Holmgren et al., 1998; Panasik et al., 1994). Hydroxylation of a few X-position prolines to 3-hydroxyproline also occurs. A few specific lysine residues in the Y-position are converted to hydroxylysine, which stabilizes extracellular crosslinking between secreted collagen molecules in the fibril.


N – TERMINAL REGION
COLLAGEN FIBRIL MONOMER
C – TERMINAL REGION
15 nm
300 nm
10 nm
Pro - α1
Pro - α1
Pro - α 2
CLEAVAGE BY
N – PROTEASE
CLEAVAGE BY
C – PROTEASE

A


ROUGH ENDOPLASMIC RETICULUM
Synthesis and modification of procollagen chains
Chain association and nucleation
Triple helix formation and secretion
INTRACELLULAR
EXTRACELLULAR
Cleavage of N- and C-propeptides
Assembly and cross-linking of fibrils

B

Hydroxylysine can undergo further modifications including glycosylations, which are thought to influence fibril structure. The timing between the α chain modifications and the triple helix assembly is crucial, for after the helix has formed the α chains are no longer accessible to modifying enzymes (Berg and Prockop, 1973).

Once the polypeptide chains have translocated to the ER lumen, the C-propeptide ends fold into a conformation that is stabilized by the formation of intrachain disulfide bonds (Doege and Fessler, 1986). It is thought that the C-propeptides anchor to the ER membrane to facilitate initial assembly (Beck et al., 1996). After the C-propeptides have associated, interchain disulfide bonds form and a nucleus of the triple helix is presented. The triple helix then continues to coil towards the amino-terminus (Bächinger et al., 1980; Kielty et al., 1993). Similar to most proteins, procollagen molecules are then secreted into extracellular space through the Golgi complex (Prockop et al., 1976).

Assembly of fibrils

The aggregation of collagen molecules into axially ordered fibrillar structures is a self-assembly process. After the chondrocytes secrete the procollagen molecules, both the N and C propeptides are cleaved by specific calcium dependent proteases, N-proteinase and C-proteinase, respectively. The N- and C- propeptides have roles in regulating fibril formation and diameter. The presence of the globular propeptides on the ends of procollagen prevents aggregation and fibril assembly from occurring intracellularly. When both propeptides have been cleaved, the processed collagen molecule is ready to be assembled into fibrils.

The functional form of collagen type II is the fibril. The reversible and entropy driven self-assembly of processed collagen molecules into fibrils is favored at body temperature. Collagen molecules are organized into fibrils in a regularly staggered pattern based on the alignment of hydrophobic and charged amino acids that overlap by approximately one quarter of their length. The amino terminus of one molecule faces the carboxyl terminus of the next, which are separated by a space. The molecules are about 4.4 times the length of the repeat period (67nm), called D. After the fibrils form they are stabilized by the formation of covalent cross-links between specific lysyl and hydroxylysyl residues. Collagen fibrils can then form ordered arrays, which further assemble into large collagen fibers that are visible under the light microscope.

As mentioned earlier, cartilage collagen fibrils are composed of collagen types II, IX and XI. Fibrillogenesis requires that each of the seven different α chains, that make up the molecules in the fibril, are correctly translated and assembled into collagen molecules. Specific enzymes required for residue modifications must also be present. For example, lysyl hydroxylase deficiency has been reported to cause Ehlers-Danlos syndrome type VI, causing hyperextensible skin and joint laxity (Wenstrup et al, 1989). In addition, the propeptides must be cleaved and fibrils are to be packaged in a tight and orderly arrangement. Considering the importance of collagen structure and the number of enzymatic steps involved in forming a collagen fibril, it is not surprising that there are many human genetic diseases that affect fibril formation.

HUMAN COLLAGEN TYPE II MUTATIONS

Chondrodysplasias are heritable disorders affecting cartilage, and in turn, the shape and the longitudinal growth of the skeleton. The clinical result of the biochemical defect may be either generalized or localized to various portions of the skeletal system. The disorders caused by collagen type II (*COL2A1*) mutations, often called type II collagenopathies, range in severity from prenatal lethal dwarfism to adults that have normal stature with premature osteoarthritis. The initial identification of *COL2A1* mutations was based on the biochemical abnormalities and how they correlated with affected skeletal tissues.

Type II Collagenopathies

Chondrodysplasias caused by *COL2A1* mutations include achondrogenesis II, hypochondrogenesis, spondyloepiphyseal dysplasia congenita (SEDc) and its variable forms, SED Strudwick, Kniest dysplasia and Stickler dysplasia (Horton and Hecht, 2000). The most severe disorders, achondrogenesis type II and hypochondrogenesis, are characterized by a short trunk with a prominent abdomen, a large head, soft cranium and a flat face. These patients die *in utero* or shortly after birth. Radiographs reveal severely underossified axial skeleton with short tubular bones. Histologically, growth plate cartilage is disorganized with an apparent increased number of chondrocytes and a reduced amount of matrix (Borochowitz et al., 1986). The cartilage collagen fibrils are thick and irregular. Interestingly, collagen type I has been found in the cartilage of these patients (Chan et al., 1995; Horton et al., 1987; Whitley and Gorlin, 1983).

SEDc is diagnosed at birth and is evident as affected individuals have a short trunk and, to a lesser extent, short limbs (Maroteaux et al., 1981; Spranger and LO Langer, 1970). Patients typically survive infancy with normal growth, yet develop a waddling gait while learning to walk. Characteristics of this disorder are quite variable with patients ranging in height from 120-140 cm (Spranger et al., 1994). Clubfoot may be present. The neck is short and the chest is barrel shaped with associated spinal problems. Although cleft palate is sometimes present, the head and facies are generally normal. Myopia with or without retinal detachment may develop. Radiographs show delayed ossification primarily of the vertebral bodies and proximal extremities (Horton and Hecht, 2000). Histological examination of the growth plate reveals shorter chondrocyte columns with collagen fibrils being irregular (Chan et al., 1993; Horton et al., 1985; Murray et al., 1989). A form of SED, SED Strudwick, appears similar to SEDc through infancy; however, it differs in childhood with increased involvement of the metaphysis where radiographs exhibit a mottled appearance (Anderson et al., 1982).

Kniest Dysplasia is usually diagnosed at birth with shortening of the trunk and extremities (Lachman et al., 1975; Spranger et al., 1994). The face of affected individuals is round and flat with midface hypoplasia and prominent eyes. Myopia, retinal detachment, cleft palate and deafness are often observed. The joints are abnormally large at birth, causing delayed motor skills. Painful joint enlargement continues into adulthood. Histologically, the cartilage is extremely soft with vacuolar degradation of both chondrocytes and matrix (Horton and Hecht, 2000). The amount of cartilage collagen fibrils are reduced (Poole et al., 1988). Most mutations reported to

cause this disorder are small in-frame deletions which result in premature translation stop signals and subsequently leads to haploinsufficiency (Weis et al., 1998).

Stickler Dysplasia primarily affects the eyes, leading to myopia and occasional retinal detachment. Cleft palate with small mandible, flat midface, hearing loss and degenerative arthritis are also observed (Stickler et al., 1965). Patients often have a slender appearance with normal height. Radiographically, irregular ossification of the epiphyses are present during childhood, while evidence of joint degeneration is observed before adolescence. Mutations in collagen type XI have been found to cause Stickler dysplasia; however, being quite rare, these disorders are not clearly defined.

COL2A1 MUTATIONS

There are over 40 heterozygous *COL2A1* mutations reported, most of which are located within the triple helical region (Kuivaniemi et al., 1997). The majority of these mutations involve amino acid substitutions (Kuivaniemi et al., 1997). Commonly, this results in the replacement of a glycine, the smallest amino acid, for a bulkier residue. For proper triple helix formation, a glycine is required to be present every third amino acid. Other mutations reported have involved in-frame deletions and duplications and are frequently associated with SED congenita. Premature stop codons, resulting from frameshift mutations, are common among Stickler dysplasias. Recurrent *COL2A1* mutations are not common; however, there are a few exceptions. Five families with late-onset SED with precocious osteoarthritis have the Arg519Cys mutation, and this has been suggested to be a mutation hot spot (Bleasel et al., 1998). Other recurrent mutations include Arg75Cys, Arg789Cys and Gly997Ser (Horton and Hecht, 2000).

The relationship between the type of mutation and the clinical manifestation is not understood and is difficult to predict. With a few exceptions, the more severe clinical phenotypes are caused by carboxyl-terminal mutations; however, there is not a strong correlation between the mutation site and a specific disorder. Quantitative differences in the amount of collagen fibrils in cartilage can explain some mildly affected patients when compared to those with more severe symptoms. This is demonstrated when the parents have somatic mosaicism with a milder phenotype than their affected child with the same mutation (Winterpacht et al., 1993). This milder phenotype is explained by the fact that in the parents, only a subset of cells produce abnormal collagen fibrils.

Most type II collagenopathies are thought to occur by a dominant negative mechanism. For instance, one *COL2A1* allele encoding a mutant α chain may prevent proper folding of the helix, and as a consequence, disturb the function of the wild-type chains. Because three α chains are needed to form a procollagen molecule and wild-type or mutant chains can be used, eight possible combinations could occur. Seven of these combinations will include at least one mutant chain. It is not understood how many mutant molecules are required to disturb procollagen assembly. However, depending on the mutation, it could be as few as one. In addition, these mutations may cause a delay in folding, allowing enzymes to overmodify residues in the unfolded chains. In most instances, the net effect is a reduction in the number of collagen fibrils in cartilage matrix.

There are several reasons why mutations in collagen molecules, and hence a reduction in cartilage collagen fibril content, could prevent proper skeletal development and growth. By reducing the number of functional collagen fibrils in cartilage, its

mechanical properties are altered. Mutant cartilage would lack structural strength needed for withstanding compressive forces on the growth plate during development or on articular surfaces surrounding the joints. A defective cartilage precursor may not provide a stable structure upon which bone matrix can be deposited during endochondral ossification. In addition, other cartilage matrix proteins may be unable to function properly without the normal number of collagen fibrils present. For example, aggrecan holds water inside the collagen fibrillar network which creates hydrostatic pressure needed to support a cartilaginous structure. Without the fibrillar network, resistance against outside forces is decreased. Furthermore, cellular events may also be altered without the proper extracellular environment.

Although these mutations often result in a cartilage matrix that has fewer, irregular collagen fibrils, the fate of the mutant α chain is variable (Prockop, 1992). Improperly folded molecules may be degraded intracellularly or retained in the rER. The procollagen molecules with mutant chains may be secreted and assembled, leading to the large irregular fibrils that have been reported. On the other hand, they may be secreted and not included in fibril assembly, making them susceptible to matrix enzymes for degradation. Any of these scenarios, could explain a cartilage matrix deficient of normal collagen fibrils.

The most obvious difficulties with studying *COL2A1* mutations in human diseases are the lack of cartilage, the difficulty of chondrocyte cultures, the inability to predict accurately the type and site of the mutation from the clinical phenotypes and the consequent need to sequence large regions of DNA to localize the mutations. Through

site-directed mutagenesis of the *Col2a1* mouse gene, transgenic mice have provided the opportunity to systematically study genotype-phenotype relationships.

MOUSE MODELS WITH *Col2a1* MUTATIONS

Chondrodysplastic mice have been produced by either disrupting endogenous *Col2a1* or by introducing a *Col2a1* transgene which expresses a mutated gene. Most of the mice with mutations in *Col2a1* develop chondrodysplasia with characteristic features including a short trunk, short thick limbs, short snout, cranial bulge, cleft palate and delayed mineralization of bone. These features are shared by lethal and severe type II collagenopathies, SED congenita, achondrogenesis and hypochondrogenesis.

The first naturally occurring *Col2a1* mutation to be detected in mice was the disproportionate micromelia (Dmm) mutant (Brown et al., 1981). These mice displayed a severe form of skeletal dysplasia, with viable heterozygotes featuring short extremities and a blunt head. Mice homozygous for the mutation die at birth due to lung hypoplasia, exhibiting a severe phenotype with extremely shortened limbs, cleft palate and abnormal cartilage structure (Foster et al., 1994). Collagen type II fibrils were greatly decreased in cartilage tissues. This mutation was the result of irradiation of mouse sperm. Linkage studies confirmed the Dmm locus to be close to the *Col2a1* locus on chromosome 15. Furthermore, sequence analysis showed the mutation to be a three nucleotide deletion within the C-propeptide. A mutation in this location probably results in decreased synthesis of procollagen molecules, and in turn, the assembly of collagen fibrils (Pace et al., 1997). A mutation in human *COL2A1*, in the region encoding the C-propeptide, has

been described for a Stickler dysplasia type I patient (Ahmad et al., 1995). The Dmm mouse has been suggested to be a model for this disorder.

The most severe phenotype was reported in mice lacking type II collagen (Li et al., 1995). By homologous recombination, *Col2a1* was inactivated when a neomycin resistance gene was inserted into exon 35. Homozygous mice die soon after birth, due to lung hypoplasia. Their extremities and trunk are very short, with interrupted cleft palate formation. The cartilage is abnormal with an extreme reduction of collagen fibrils and a disorganized epiphyseal growth plate. In addition to the limb bones being short, they feature thick cortical bone collars and lack bone marrow. The resulting phenotype strongly suggests that collagen type II is required for endochondral ossification and for maintaining the strength of the cartilage matrix. The severe phenotype associated with a lack of collagen type II has been comparable to the lethal human chondrodysplasia, achondrogenesis type II (Chan et al., 1995).

Many transgenic mice have been generated which result in different forms of chondrodysplasias, ranging from severe to mildly affected skeletal phenotypes. To test whether the assembly of collagen fibrils is disturbed by overexpressing *Col2a1*, a transgene encoding the entire wild type gene sequence was used to generate transgenic mice. Mice, with the highest expression, had large abnormal fibrils and died at birth (Garofalo et al., 1993). However, unlike other mice with collagen type II mutations, they did not have cleft palate, abnormal facial or skeletal features with the exception of slightly shorter extremities. Although the skeleton appears primarily normal, the cartilage contains abnormally thick collagen fibrils, perhaps resulting from an excess of collagen type II compared to types IX and XI.

Mice expressing transgenes with internal deletions also have a lethal chondrodysplastic phenotype. Two transgenic mouse lines were generated to have deletions: (1) by expressing an internally deleted human *COL2A1* minigene and (2) by expressing murine *Col2a1* with a deletion in exon 7 (Metsäranta et al., 1992; Vandenberg et al., 1991). Each study resulted in a severe short-limbed dwarfism, a short trunk and snout, a protruding tongue and cleft palate. The epiphyseal growth plates were disorganized with a decrease in cartilage collagen fibrils. However, the minigene was lethal in only 15% of the mice; the remaining heterozygotes were viable with mild chondrodysplastic features developing symptoms of arthritis. After 15 months, these animals had degenerative changes in the articular cartilage, similar to that seen in human osteoarthritis.

To model *COL2A1* mutations found to cause human disorders, transgenic mice have also been generated with point mutations within the triple helical domain. Transgenes encoding the Gly85Cys and Gly574Ser mutations resulted a lethal phenotype with extreme abnormalities in mice (Garofalo et al., 1991; Maddox et al., 1997). These shared severe features of chondrodysplasia similar to other mice described above, including shortened long bones and a disorganized epiphyseal growth plate. The number of collagen fibrils were reduced, which probably explains the common relationships between phenotype and the different collagen type II mutations.

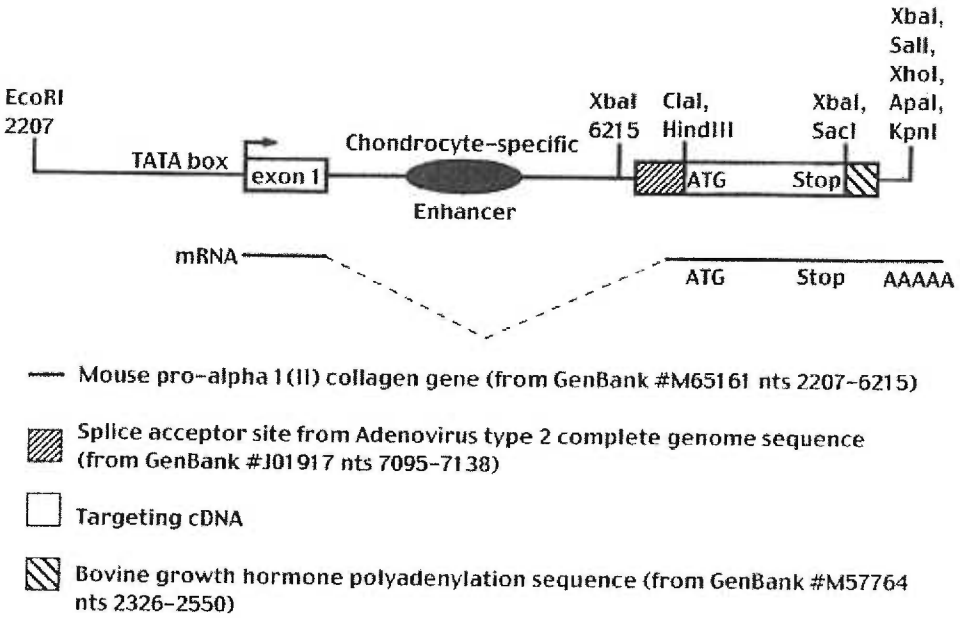
To better understand the functions of other cartilage matrix proteins and signaling molecules, it has been convenient to use the *Col2a1* promoter to govern expression of transgenic constructs (Mukhopadhyay et al., 1995; Zhou et al., 1995). Because *Col2a1* is expressed highest in chondrocytes, a chondrocyte-specific expression vector has been

engineered, by altering the *Col2a1* promoter and intron 1 (Figure Intro-3), to allow gene targeting in transgenic mouse chondrocytes (Garofalo and Horton, 2000). With this tool available, other transgenic lines have been generated which have proven to be good models of human skeletal diseases (Garofalo et al., 1999).

Transgenic mouse technology is an extremely powerful method to analyze biological processes and pathogenesis of disease. Mouse models have been important for deciphering the mechanisms by which mutations, and their corresponding dysfunctional proteins, cause specific phenotypes. The availability of mouse models of human diseases offers us an experimental system to monitor disease progression and to test potential therapeutic methods.

STRATEGIES FOR THERAPEUTIC INTERVENTION

The ability to insert or delete genetic material in transgenic animals provides unique opportunities to dissect the role of specific genes and to evaluate novel therapeutics. Animal models of chondrodysplasias allow the evaluation of new treatments to correct or decrease the effects of mutations. Most strategies have involved the replacement of functioning genes for nonfunctional ones. This approach has been particularly successful for loss-of-function mutations involving enzyme deficiencies. However, the addition of functional genes for defective genes will probably not work for mutations that act through dominant negative mechanisms, like most *COL2A1* mutations. Alternate therapeutic agents have been developed to discriminate between sequences, DNA or RNA, to block the expression of mutant transcripts. Antisense technologies


EcoRI
2207
TATA box
exon 1
Chondrocyte-specific
Enhancer
XbaI
6215
ClaI,
HindIII
ATG
XbaI,
SacI
Stop
XbaI,
SalI,
XhoI,
ApaI,
KpnI
mRNA
ATG
Stop
AAAAA
Mouse pro-alpha 1(II) collagen gene (from GenBank #M65161 nts 2207-6215)
Splice acceptor site from Adenovirus type 2 complete genome sequence (from GenBank #J01917 nts 7095-7138)
Targeting cDNA
Bovine growth hormone polyadenylation sequence (from GenBank #M57764 nts 2326-2550)

could potentially treat genetic disorders resulting from heterozygous dominant negative acting mutations.

Strategies to selectively block gene expression are based on the fact that for a gene or its mRNA to function properly, its nucleotide sequences must exist at least transiently in an exposed, single stranded state (Wagner, 1994). If these exposed sequences were to base pair with a complementary sequence, an antisense sequence, the expression of the gene could be inhibited. Antisense (AS) nucleic acids that can be specifically designed to inhibit gene expression can take the forms of short AS oligodeoxynucleotides, AS oligodeoxyribonucleotides or ribozymes (Muotri et al., 1999).

Antisense Oligodeoxynucleotides and Oligodeoxyribonucleotides

Antisense oligos (ASOs) have been used successfully to regulate eukaryotic gene expression in a wide variety of cell-free, cell culture and whole animal model systems (Izant and Weintraub, 1985; Joshi et al., 1991; Simons et al., 1992; Khillan et al., 1994). Short oligos, 10-20 bases, are taken up by cells and are sufficient to block gene expression in a highly specific fashion. Inhibition of gene expression relies on the ability of an ASO to bind a complementary sequence and prevent translation of the mRNA. RnaseH hydrolyses the RNA strand of a RNA-DNA duplex and is likely to be responsible for the antisense effects. The mechanism of inhibition include degradation of RNA/DNA hybrids by RnaseH, interruption of nuclear processing of mRNA transcripts and arrest of translation. The greatest success has been achieved by directing antisense oligos against translation initiation or splice junction regions.

A new potential antisense technique involves the use of double-stranded RNA (dsRNA) to block gene expression (Guo and Kemphues, 1995). It has been shown that dsRNA may interfere with gene activity more strongly than single-stranded RNA (Fire et al., 1998). The interference effect of this dsRNA, called RNA interference, is remarkably long-lived and may cross cell tissues or even affect an animal's progeny (Tabara et al., 1998). This tool has been employed for *C. elegans* studies, but remains to be investigated in mammals.

Triplex DNA strategies also aims to block production of an undesired protein by selectively inhibiting transcription. In this case, oligos are designed to bind to double-helical DNA. The power of this approach lies mainly in the extreme specificity of the interaction between the oligos and the duplex DNA target. The triplex-forming oligo establishes two new hydrogen bonds with purine bases in the targeted region of the duplex (Frank-Kamenetskii and Mirkin, 1995; Chan and Glazer, 1997). However, to form the triple helix, there is a need for a purine-rich region on the duplex target. Otherwise, the oligo will fail to provide a stable triplex binding in the DNA target. This requirement limits the use of this method (Radhakrishnan and Patel, 1994).

One major problem with antisense oligos is their inability to cross cellular membranes efficiently. They can accumulate in the nucleus if microinjected into the cytoplasm; however, when added to the culture medium the oligos tend to accumulate in cytoplasmic granules, probably endosomes and lysosomes, but not the nucleus. Additional cell permeabilization techniques, cationic liposomes, must be utilized to enhance cytoplasmic and nuclear localization. Another disadvantage is that different ASOs will have variable binding affinities. In the presence of a normal sequence,

antisense specificity for a mutant sequence depends on the number of base differences between these two sequences. Hence, ASOs bind to mutant targets with greater selectivity when there are more differences. When targeting a sequence with a single base substitution, ASOs may recognize the mutant and normal sequence equally preventing transcription of both sequences.

Ribozymes

Ribozymes are trans acting RNA molecules that cleave RNA in a sequence-dependent fashion. Their catalytic domains have been adapted from self-splicing plant viroid and virusoid RNAs (Cech et al., 1981; Guerrier and Altman, 1984). Binding of the mRNA target to the ribozyme is based on strand complementarity. Thus, any inhibitory effect seen may include a contribution resulting from this antisense character, in addition to the catalytic effect of the ribozyme. Ribozymes offer significant advantages over ASO approaches for gene inhibition in mammalian cells.

When compared to ASOs, the binding of ribozymes to their target mRNAs is more stringent and more stable, reducing nonspecific effects often associated with other antisense approaches (Stein, 1995; Wagner, 1995). In addition, a single ribozyme molecule can cleave many RNA target transcripts. This allows these enzymes to be used at much lower concentrations than ASOs with much lower toxicity. Among the several classes of ribozymes, the hammerhead ribozyme is probably best understood. The name "hammerhead" was given due to the secondary structure of the self-cleaving domain (Symons, 1992).

Hammerhead ribozymes can be synthesized as relatively short RNA molecules containing the core catalytic sequence flanked by short complementary sequences that direct the ribozyme to the RNA transcript. The catalytic core consists of a consensus sequence of 14 nucleotides. The specificity of the ribozyme to its substrate is conferred by base pairing of flanking sequences with those of the target RNA. The cleavage site on the target RNA is defined by the trinucleotide sequence XU, A, C or U. Cleavage occurs at the 3'side of this sequence (Figure 1-2).

The mechanism of ribozyme catalysis can be summarized in three steps: substrate interaction, cleavage and dissociation (Fedor and Uhlenbeck, 1992; Hertel et al., 1994). The cleavage rate reflects the rapidity of phosphodiester bond cleavage and may be affected by salt concentration, pH and temperature. One complication is that ribozyme activity requires a higher Mg^{2+} concentrations than is estimated to be present in most cellular environments; therefore, their functions *in vivo* may be limited due to the physiological concentration (Wang and Ruffner, 1998). Another disadvantage is that the association rate of ribozymes to long substrates (mRNAs) may be slowed or totally prevented due to the presence of secondary structures. Anti-gene therapy by ribozymes has attracted a great deal of attention and efforts are being made to improve the properties of these molecules.

RESEARCH GOALS

My thesis work has focused on analyzing the skeletal consequences of two transgenic mouse lines with mutations in type II collagen or cartilage oligomeric matrix protein. The first transgenic experiment was to model a *COL2A1* mutation found to cause spondyloepiphyseal dysplasia congenita (Chan et al., 1993). The single base change replaced a C for a T in exon 41, which resulted in an arginine to cysteine substitution at residue 789. The altered amino acid is in the Y position of the Gly-X-Y repeats where changes are usually tolerated. The human pathology suggested that the R789C transgenic mouse would have a phenotype severe enough to recognize and yet mild enough to allow reproduction. The transgenic skeletal phenotypes were analyzed by cleared staining, histological procedures, immunohistochemistry and electron microscopy. Transgene expression and protein production were also analyzed. The ultimate goal was to define the mechanism of the mutation and to develop a ribozyme based gene therapy for the mutant transcript.

Secondly, transgenic methods were used to study the biological function of cartilage oligomeric matrix protein (COMP). COMP is a pentameric glycoprotein found abundantly in cartilage, tendon, ligament and synovium. Each COMP pentamer is composed of five identical monomers, which are assembled by a coiled-coil domain. By using the collagen type II promoter (Figure Intro-3) to overexpress the assembly domain in cartilage, COMP molecules should be produced which have a mixture of normal and shorter arms. In an attempt to understand the function of COMP, transgenic mice were generated which were hypothesized to have a disruption in normal COMP assembly. The transgenic skeletal phenotypes were analyzed by cleared staining, radiography, growth

measurements, histological procedures, immunohistochemistry and electron microscopy. Protein analysis was also performed. My hypothesis was that pentamerization is required for normal COMP function; therefore, by inhibiting pentamerization one could gain insight into the function of COMP in skeletal development.

MATERIALS AND METHODS

TYPE II COLLAGEN R789C MUTATION

Construction of the Transgene

Two mutations were introduced by PCR mutagenesis into the mouse type II collagen gene, *Col2a1*. Cosmid pWE15, which contains the murine wild-type *Col2a1*, has been described (Garofalo et al., 1991) and used to produce several transgenic mouse strains (Garofalo et al., 1993; Garofalo et al., 1991; Maddox et al., 1997; Metsäranta et al., 1992) (Figure Intro-4). The first mutation, a C to T transition in exon 41, resulted in the amino acid substitution, Arg789Cys. The second was a silent mutation that created a *SmaI* cleavage site in exon 41. The sense strand primer (5'-AGCTGGTGACCCCG GTCTTGAAGGT-3') contains a *BstEII* cleavage site. The anti-sense primer (5'-ACC GATGGGCCGGGAAGGCCGGGGAATCCTCTCTCACCACACTGCCCGGG-3') contained the *SfiI* restriction site and three single base changes, resulting in the amino acid substitution of interest and the silent mutation used to monitor the presence of the transgene and its RNA transcripts. The DNA fragment was amplified using a *ClaI-XbaI* subclone of the wild-type mouse *Col2a1* as a template. After PCR mutagenesis was performed, the product was ligated into the original cosmid vector, pWE15, using the *BstEII* and *SfiI* cleavage sites.

The transgene was removed from the vector by *NotI* digestion. This fragment was microinjected into one-cell mouse embryos, derived from B6D2F_1 matings. The embryos were implanted into CD1 pseudopregnant female mice. The founder mice were mated with C57BL/6J mice to produce F_1 litters. To identify founder mice and determine

transgene copy number, genomic DNA was isolated from tails and digested with *NcoI*. A previously introduced silent mutation that disrupts an *NcoI* restriction site in exon 7 of the transgene allowed the endogenous and transgene *Col2a1* fragments to be distinguished on Southern blot using an *EcoRI-PstI* fragment from intron 6 to intron 8 as a probe (Garofalo et al., 1991). This probe hybridizes to fragments (540bp and 628bp) in the endogenous type II collagen gene and to a fragment (1168bp) in the transgene. Transgenic embryos were similarly genotyped using genomic DNA isolated from placentas (Garofalo et al., 1991).

Microscopy

Cesarean section was performed at 18.5-19.5 days p.c. to obtain fresh tissue samples. After skin and internal organs were removed, the skeletons were cleared in 1% KOH and stained with alcian blue and alizarin red S (Maddox et al., 1997; McLeod, 1980). Histology was performed on hind limbs that were fixed in 4% buffered formaldehyde, embedded in paraffin, sectioned and stained with hematoxylin, eosin and alcian blue. Immunohistochemistry was carried out on paraffin sections using polyclonal antibodies against collagens types II and X and aggrecan. The type II collagen antibody was obtained from Southern Biotech (Catalog number 133-01), the type X collagen antibody from G Lunstrum (Portland, OR) and aggrecan antibody from K. Doege (Tampa, Fl). The sections were then treated with a fluoresene-labeled secondary antibody. The epiflourescence was visualized by Zeiss, Autophot.

Forelimb specimens, from 16.5 dpc embryos, were dissected for electron microscopy, fixed in 1.5% glutaraldehyde/1.5% paraformaldehyde and 1 % osmium tetroxide, dehydrated in ethanol and embedded in Spurr's epoxy resin. Ultrathin sections were examined on a Philips 410-LS transmission electron microscope. Immunoelectron microscopy was performed using antibodies specifically to collagens type II (Fisher Biotech), type IX and type XI (20545) as described previously (Keene et al., 1995).

Quantitation of *Col2a1* mRNA Transcripts

Total RNA was extracted from mouse limbs (Trizol method, Gibco, BRL) and amplified by RT-PCR using primer sets that recognized two different regions of the type II procollagen transcript (Garofalo et al., 1993; Garofalo et al., 1991; Metsäranta et al., 1992). Primers A4 (5'-AACTTCGCG GCTCAGATGGCTG-3') and A5 (5'-CAGAG ACACCAGGCTCGCCAGGTTC-'3) amplified a fragment containing the *NcoI* cleavage site that was present in the wild type but absent in the transgene sequence. A second primer set flanked exon 41 sequences containing the *SmaI* site present only in the transgene. The primers were Ex-40 (5'-AAAGGTGTTCGAGGAGACAGTGGC-3') and Ex-42-inv (5'-ACCTCTGTCTCCAGACGCGCCAGG -3'). Radiolabeled ^{32}P-dCTP was added to the PCR reactions to detect the cDNA fragments. The PCR products were digested with their respective enzyme, *NcoI* or *SmaI*, and the fragments were separated by electrophoresis. The relative amounts of transgene and endogenous *Col2a1* derived transcripts were then estimated from visual inspection and confirmed by Betagen densitometry of the electrophoretic patterns. Transgene expression was similarly monitored in the stably transfected Swarm rat chondrosarcoma cell lines.

Analysis of Type II Collagen Synthesized by Cells in Culture

The construct used to generate transgenic mice was stably transfected into Swarm rat chondrosarcoma cells by calcium phosphate precipitation (Chen and Okayama, 1987). Plated at 1 x 10^6 cells per well in a 6 well dish, untransfected and transfected cells were cultured in the presence of DMEM, FCS (10%), ascorbic acid (50 μg/ml) and 4-aminopropionitrile fumarate (50 μg/ml) for 24 hours. After incubation, the culture medium was harvested and the cell/pericellular matrix layer was solubilized in 0.25M NH_4OH. The noncollagenous proteins and nonhelical regions of the collagen molecules were digested with 0.2mg of pepsin/ml in 0.5M acetic acid overnight. The samples were then dialyzed against 25 volumes of 100mM Tris-HCl, pH 8.0 and then 4 times against water. The collagenous proteins were recovered by lyophilization and separated by 6% PAGE under nonreduced conditions. Following electrophoresis the resolved proteins were transferred to a PVDF membrane in 10mM CAPS buffer. The membrane was incubated with a mouse monoclonal antibody to collagen type II (Neomarkers, Inc., collagen type II, Ab-2, Clone 2B1.5). HRP-conjugated secondary antibodies were detected by chemiluminescence (Pierce Super Signal). Untransfected and transfected cells were also labeled with 50 μCi/ml ^{35}S-cysteine. The cells were incubated and fractions (pericellular matrix, cell lysate and medis) were prepared by standard methods. The protein samples were analyzed by 6% SDS-PAGE and viewed by autoradiography.

Ribozyme Constructs

To test ribozyme activity *in vitro*, two target constructs, wild-type and mutant were generated (Figure 1-2). By using overlap extension PCR, two fragments were obtained. The wild-type fragment was amplified by using primers KSG1 (5'-AGGAAG CTTGATGGGAGTCATGCAAGGGCCCATGGGCCC-3') and KSG2 (5'-TTTGAA TTCCCTGGGGGTCCACGGGGTCCCATGGGGCCCATGGGC-3'). The mutant fragment, containing the ribozyme cleavage site, was amplified by using primers KSG1 and KSG3 (5'-TTTGAATTCCCTGGGGGTCCACGGGGTCCCATTGGGCCCATGG GC-3'). Each fragment was cloned into pSP65 vector (Stratagene, Inc.), using *EcoRI* and *Hind III* restriction sites and sequenced for confirmation.

The ribozyme construct was made similarly, using overlap extension PCR. The primer set KSG4 (5'-AGGGAATTCTAATACGACTCACTATAGTATCTACCACGG GGTCCCACTGATGAGTCCGTGAGG-3') and KSG5 (5'-TCTAAGCTTAATTAAC CCTCACTAAAGATCTATGGCCATGGGCCCTTTCGTCCTCACGGACTCATCAGT GGG-3') were used to generate the fragment. The fragment was cloned into pSP65 vector and sequenced for confirmation.

***In vitro* transcription**

Each of the target constructs were digested with *Hind III*, then *in vitro* transcription was performed using SP6 polymerase (Ambion MAXIscript Kit, Ambion, Inc). Each target was labeled with 10μCi α-^{32}P UTP (Amersham, Inc.). For transcription of the active ribozyme, the ribozyme construct was digested with *Hind III*, and initiated

by the T7 polymerase. For the inactive ribozyme, the construct was cleaved with *Eco RI*, then this sequence was transcribed in reverse using the T3 polymerase. Neither the active or the inactive ribozyme transcripts were radioactively labeled. All transcripts were gel purified. The gel slices were crushed in RNA extraction buffer (0.5M sodium acetate, 0.5 mg/ml SDS, 2mM EDTA) and vortexed for 4 hours in cold room (12C). The RNA was then precipitated with ethanol and sodium acetate at –20C overnight.

Ribozyme Cleavage Reaction

The reactions were carried out in 20µl volume. Ribozyme and target RNA transcripts, 1 pmol of each, were combined. They were heated to 95C for 2 minutes then quickly put on ice. $MgCl_2$ was added to initiate the reaction (Chang et al., 1990). Final concentrations of the reactions were 50mM Tris-HCl, pH 7.5, 20mM $MgCl_2$, and 1mM Na-EDTA. The reactions were overlaid with mineral oil to prevent evaporation. Experimental mixtures were incubated for 4 hours at 37C. Reactions were stopped by adding 0.2 vol 200mM Na-EDTA, pH 7.9. When optimizing the reaction conditions, the amount of ribozyme, target and $MgCl_2$ were altered, as well as the incubation time and temperature. Reactions were run on 15% polyacrylamide gels. The gels were dried and exposed to X-O-MAT Blue radiography film (Kodak).

TRUNCATED-COMP ASSEMBLY MUTATION

Construction of the transgene

Total mouse RNA was isolated from newborn limb tissue (Trizol, Gibco BRL, Inc.). Using RT-PCR, the coiled-coil domain was amplified from the cDNA reaction, using primers based on the rat COMP gene sequence. The mouse COMP gene sequence was not available. Using primers COMP-ATG (5'-GCAGCTCCGCCGCCATGGGCCCCAC-3') and COMP-300 (5'-GGGAAGCAGGAGCCGGGTGCGCAG-3'), a 303bp fragment was generated. Then, using the COMP-ATG and COMP-STOP primer (5'-GCGCCATGGTCAC ACGCTCAGACCGGGGGTGCGTGCG-3'), a stop codon was incorporated into the sequence. The coding sequence is 252bp (84 residues), which encode the signal peptide and the coiled-coil domain, including the terminal cysteines for added stability (Appendix 2). Using *Hind III* and *Eco RI* restriction sites, the fragment was subcloned into pBluescript (Stratagene) vector and sequenced. The correct fragment was then cloned into the pCDNA3 vector (Invitrogen) for possible use in tissue culture. Because there wasn't a convenient restriction site that would allow removal of the pCDNA3 polyadenalation sequence, a second polyadenalation sequence (from bovine growth hormone) was inserted using the *EcoRI* and *XhoI* restriction sites. Finally, using a *HindIII* and *XhoI* restriction digest this fragment, containing the coiled-coil domain and the bovine polyadenalation sequence, was removed from the pCDNA3 vector and inserted into the mouse *Col2a1* expression vector, SP-βgeo-BpA (Figures Intro-3, 2-3) (Garofalo and Horton, 2000.

The transgene was removed from the vector by *NotI* digestion. This fragment was microinjected into one-cell mouse embryos, derived from B6D2F_1 matings (DNX Transgenic Facility, Cranbury, New Jersey). The embryos were implanted into CD1 pseudopregnant female mice. The founder mice were mated with C57BL/6J mice to produce F_1 litters. To identify founder mice and determine transgene copy number, genomic DNA was isolated from tails and digested with *EcoRI*. The coiled-coil domain was used as a probe for Southern analysis, which identified the transgene (600bp) and a much larger endogenous fragment. Founder copy number was quantitated by using STORM phosphorimager (Molecular Dynamics, Inc.) and Image Quant Software for analysis. Transgenic offspring were similarly genotyped using genomic DNA isolated from placentas (Garofalo et al., 1991). PCR was also used to genotype the offspring using a primer set specific to the transgene, Col2Xba (5'-GGAGAGGGTCCAGCCGG AGCTAC-3'), and the COMP-STOP primer (5'-GCGCCATGGTCACACGCTCAGA CCGGGGGTGCGTGCG-3').

Microscopy

Offspring were sacrificed at birth or after Cesarean section performed at earlier stages to obtain fresh tissue samples. The tails or placentas were used for genotyping. After skin and internal organs were removed, tissues were cleared in 1% KOH and stained with alcian blue and alizarin red S (Maddox et al., 1997; McLeod, 1980). Histology was performed on hind limbs and fore limbs that were fixed in 4% buffered paraformaldehyde or 10% buffered formalin, embedded in paraffin, sectioned and stained with hematoxylin, eosin and alcian blue. Immunostaining was carried out on paraffin or

frozen sections using polyclonal antibodies against COMP, collagen type II and type X. The mouse monoclonal type II collagen antibody was obtained from Neomarkers, Inc. (Ab-2, Clone 2B1.5), the type X collagen antibody from G Lunstrum (Portland, OR) and the polyclonal human COMP antibody was obtained from L Sakai (Portland, OR). Fore limb specimens for electron microscopy were dissected immediately after birth, fixed in 1.5% glutaraldehyde/1.5% paraformaldehyde and 1 % osmium tetroxide, dehydrated in ethanol and embedded in Spurr's epoxy resin. Ultrathin sections were examined on a Philips 410-LS transmission electron microscope.

Adult Mouse Skeletal Studies

Transgenic and normal offspring were allowed to age to 5 months. They were weighed and X-rays were taken to measure spine and femur length. The data (see Appendix 2) were used to determine if there is a correlation between being transgenic and having smaller skeletal features. Offspring from five different founders were used. There were 191 mice in all; 46 transgenic females, 45 normal females, 43 transgenic males, 57 normal males. The data were analyzed using the SYSTAT (version 8.0 for windows) program (SPSS, 1998). Values for minimum, maximum and the range (difference) between these data points were noted. Calculations for the median, mean, standard deviation, variance, and Pearson X^2 were obtained. The data were analyzed by individual founder line and a combination of lower copy number and higher copy number groups.

Analysis of COMP Synthesized by Rib Chondrocytes in Culture

Rib cages were dissected from wild-type and transgenic newborn littermates. Following pronase and collagenase D digestion, chondrocytes were isolated. The chondrocytes were washed with PBS and cultured at 37C overnight on DMEM with 15% fetal bovine serum, 50μg/ml Ascorbic Acid and 10ng/ml TGFβ-1. The chondrocytes were again washed and cultured overnight on DMEM with decreased serum (0.5%). The following day, media and cell fractions were collected, and protease inhibitors (Sigma protease cocktail II, P8340, and DFP) were added. The samples were dialyzed against a Tris-HCl/NaCl solution and collected. The cell and media protein fractions were analyzed by 4.5% PAGE under non-reduced conditions. Following electrophoresis the proteins were transferred to a PVDF membrane in 10mM CAPS buffer. The membrane was incubated with a human polyclonal antibody to COMP (L Sakai, antibody 3593). HRP-conjugated secondary antibodies were detected by chemiluminescence (Pierce Super Signal).

CHAPTER 1

A Non-glycine Type II Collagen Mutation Disrupts Skeletal Development in Transgenic Mice

BACKGROUND

Heterozygous mutations of *COL2A1* are associated with the spondyloepiphyseal dysplasia (SED) category of human chondrodysplasias. SED is a heterogeneous group of disorders characterized by a short trunk with or without short limbs and flattened vertebral bodies (Cole, 1994). When these characteristics are present at birth and accompanied by a short neck, a barrel-shaped chest and rhizomelia, but normal hands and feet, the diagnosis is SED congenita (Horton and Hecht, 2000) (Figure 1-1A). SED congenita has a moderately severe clinical phenotype. Cleft palate, severe myopia and retinal detachment have also been documented in these patients. X-rays have revealed a delay in ossification, especially of the femoral heads, decreased vertebral body height, flaring of ribs, bowed femoral shafts and an irregular epiphyses.

R789C MUTATION

A 4 year-old patient with moderately severe SED congenita has been reported to carry a heterozygous mutation in *COL2A1* (Chan et al., 1993). The single base change replaced a C with a T in exon 41, which resulted in an arginine to cysteine substitution at residue 789 in the triple helical domain (Figure 1-1B). Collagen type II from patient cartilage and cultured chondrocytes showed that approximately one third of the pro α-chains were mutant. This suggested that secretion of the procollagen molecules containing the mutant chains was inhibited. In addition, α1(II) chains extracted from patient cartilage contained disulfide-bonded dimers. Since then, this mutation was identified a second time in an unrelated child (Chan et al., 1995).

Of the over 40 mutations in *COL2A1* reported to date, about half have been missense mutations in which a glycine is replaced by an amino acid with a bulkier side chain (Kuivaniemi et al., 1997). When α chains containing nonglycine residues in the Gly position are incorporated into a triple helix, they disrupt helix assembly due to slower folding and increased glycosylation (Byers et al., 1991). Substitutions at the X and Y positions are expected to behave differently since the extension of a bulky side chain outward from the axis should not distort the helix proper. An exception is if the substitution introduces a cysteine residue creating the opportunity for disulfide bonds to form and in turn disrupt fibril assembly.

The arginine for cysteine substitutions are of special interest because they are the first nonglycine substitutions shown to cause a phenotype. They are the most frequently recurrent mutations in *COL2A1* (Kuivaniemi et al., 1997). Arginine to cysteine substitutions have been reported in COL2A1 at Y-positions 75, 519 and 789 (Ala-Kokko et al., 1990; Bleasel et al., 1995; Horton and Hecht, 2000; Williams et al., 1993). The first two are associated with relatively mild SED phenotypes, whereas the manifestations were moderately severe in the third. Recently, a recurring arginine to cysteine substitution in the X position of COL1A1 was reported to cause Ehlers-Danlos syndrome (Nuytinck et al., 2000). These mutations show that X and Y position mutations are also important for fibrillar collagen function.

The analysis of tissue collagen extracts from a patient with the R789C mutation and cell culture studies of recombinant type II collagen containing the R519C mutation has suggested that such substitutions lead to intermolecular disulfide bonds (Chan et al., 1993; Fertala et al., 1997). These studies reported that the greatest adverse effect was on

the assembly of type II collagen-containing fibrils. In addition, Fertala et al showed that recombinant collagen molecules comprised of mutant monomers did not form fibrils and disrupted the fibrillar assembly of normal collagen molecules (Adachi et al., 1999; Fertala et al., 1997). These observations implicate defective fibrillogenesis as a major component in the pathogenesis of this group of chondrodysplasias.

EXPERIMENTAL STRATEGY

The generation of the R789C transgenic mice was designed to meet three goals. The first was to gain insight into how this mutation disrupts bone growth. This was the first transgenic mouse to model a collagen Y- position amino acid substitution. Mouse tissues were analyzed by immunohistochemistry and electron microscopy to determine the composition of affected cartilage. The second goal was to determine how cartilage collagen fibrillogenesis is affected by this type of mutation. The presence of a cysteine in the triple helix where there is normally no cysteine residues, allowed the mutant collagen chains to be studied biochemically. Thirdly, the model was intended to be used to determine the effectiveness of correcting a dominant negative mutation through antisense therapeutic interventions. Silent mutations inserted in the transgene for genotyping creates a ribozyme cleavage site which made the mouse model suitable for testing therapeutic strategies *in vivo*.

Construction of the transgene

The transgene, governed by the murine *Col2a1* promoter, encodes the murine *Col2a1* gene with the R789C mutation. The construction of the mouse mutation is shown

in figure 1-1C. A DNA fragment, containing the mutation site, was amplified using a *ClaI-XbaI* subclone of the wild type mouse *Col2a1* as a template. Primer 1 spans a *BstEII* restriction site, while primer 2 spans a *SfiI* site. Primer 2 contains three single base changes. The first change creates the R789C mutation and a ribozyme cleavage site (filled circle, Figure 1-1C). The second and third mutations are silent mutations, which did not change the amino acid residues but introduced a *SmaI* restriction site that was used to monitor the mutant transcripts (square, Figure 1-1C). These mutations also provided additional base differences between the wild type and mutant mRNA sequences that were intended for antisense recognition. The *BstEII-SfiI* fragment was then cloned into a *ClaI-XbaI* fragment and assembled into the murine *Col2a1* as previously described (Maddox et al., 1997). Another silent mutation, inserted for past experiments, eliminates the wild type *NcoI* site in exon 7 providing a second site to determine transgene expression levels and another ribozyme cleavage site. The ribozyme construct was made to target this site (Materials and Methods).

Antisense therapy

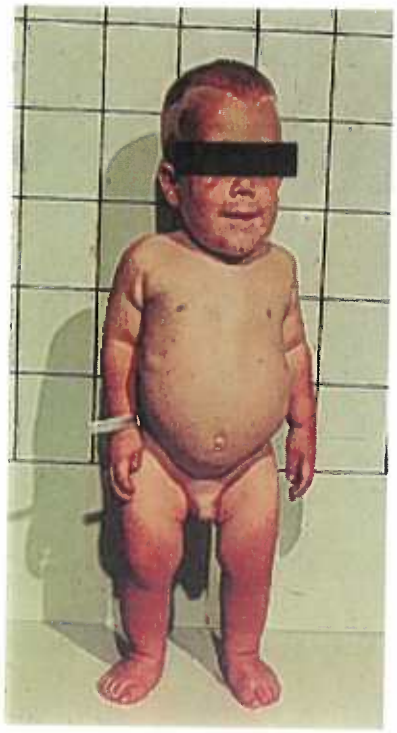
Ribozymes are catalytic, *trans* acting RNA molecules that cleave other RNA molecules (Cech and Uhlenbeck, 1994). The specificity of the ribozyme to its target RNA is conferred by base pairing of flanking sequences with those of the target RNA (Figure 1-2A). The hammerhead ribozyme cleavage site on the target RNA is defined by the trinucleotide sequence XUA, XUC or XUU, where X is any nucleotide. The cleavage site in this experiment is CUA as underlined in Figure 1-2A. Cleavage will then occur at the 3' side of this sequence. Hammerhead ribozymes have two important advantages

over conventional antisense oligonucleotides. First, the cleavage of the target mRNAs is much more effective at disabling the targeted transcripts than base-pairing alone. Secondly, as an enzyme a single ribozyme molecule can cleave many RNA target transcripts which allows ribozymes to be used at much lower concentrations and, therefore, reduces the toxicity imposed on the cells.

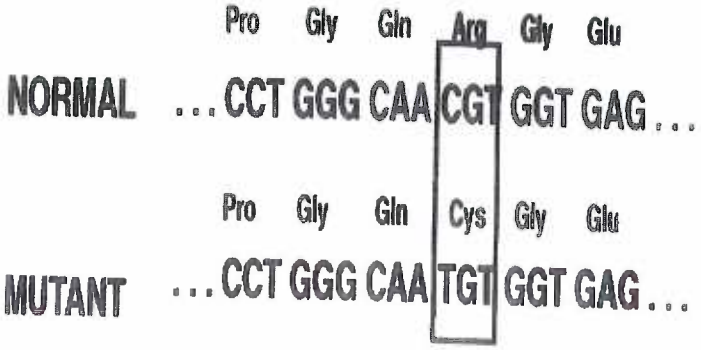
The ultimate goal of this study was to generate transgenic mice which would express a ribozyme, in a cartilage specific manner, that is targeted to the R789C mutant transcript. Through breeding a ribozyme founder mouse with a collagen type II--R789C founder mouse, the pups carrying both transgenes would be analyzed to determine the therapeutic effect of the ribozyme on skeletal development. These "double" transgenics were to be compared to the wild-type and R789C mutant littermate phenotypes.

It is important to note that the change from a dominant negative *COL2A1* mutation to a null allele may not result in complete reversal of the phenotype. Heterozygous point mutations that cause premature stop codons within the triple helical domain have been reported in Stickler dysplasia (Horton and Hecht, 2000). This situation is similar to a having one wild type allele and one null allele. However, the Stickler phenotype is milder than most SED phenotypes associated with *COL2A1* mutations. This ribozyme approach could at least decrease the severity of the disorder.

Figure 1-1. SED congenita and the R789C mutation. (A) A SEDc patient, four years old, featuring short limbs, a short trunk and a barrel-shaped chest. (B) The *COL2A1* mutation found to cause SEDc in another patient (Chan et al., 1993). The single base change replaced a C with a T in exon 41. This resulted in an arginine to cysteine substitution at residue 789 in the Y-position of a Gly-X-Y repeat. (C) The construction of the R789C transgene. The region, in *Col2a1*, surrounding the mutation was amplified by primers 1 and 2. Primer 2 contains an alternative sequence, resulting in the C to T transition (filled circle), which generates the R789C mutation. Primer 2 also contains two silent mutations (empty squares) which introduces a *SmaI* site used to monitor transgene expression. After the sequence change was confirmed, the fragment was cloned back into the murine Col2a1 gene using the *BstEII* and *StiI* restriction sites. A *NotI* digestion was performed to linearize the transgene before pronuclear injection.



A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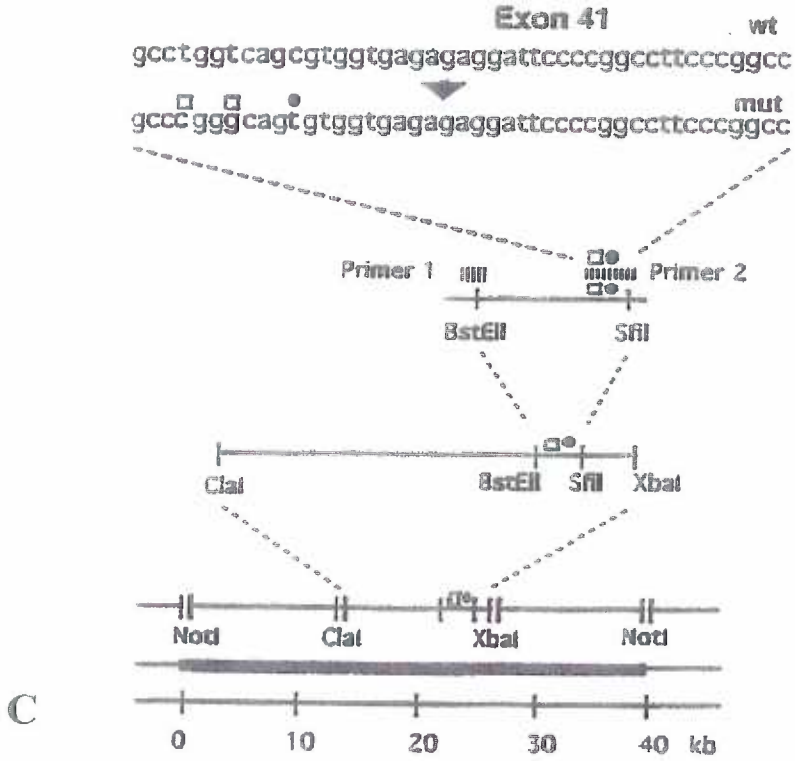
Pro Gly Gln Cys Gly Glu
MUTANT ...CCT GGG CAA TGT GGT GAG...

B


Exon 41
wt
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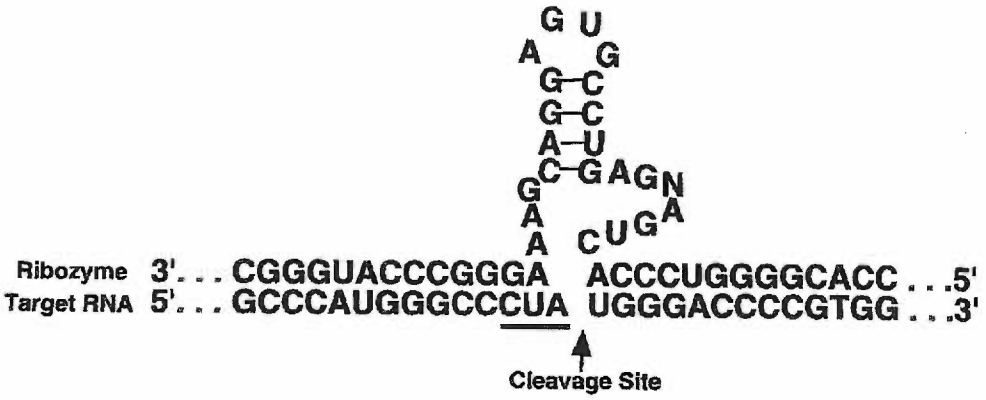
mut
gcccgggcagtgtggtgagagaggattccccggccttcccggcc
Primer 1
Primer 2
BstEII
SfiI
ClaI
BstEII
SfiI
XbaI
NotI
ClaI
XbaI
NotI
0
10
20
30
40
kb

C

Figure 1-2. Ribozyme model and *in vitro* cleavage experiments. (A) Ribozyme and mutant RNA target sequences at the site of cleavage (underlined CUA). (B) *In vitro* ribozyme cleavage experiment showing wild-type and mutant full length transcripts (180bp) and two cleavage products (111bp) and (69bp). Lanes 1 (wt) and 2 (mut), control experiment without ribozyme. Lanes 3 (wt) and 4 (mut), experiment with ribozyme (1 pmol) and $MgCl_2$ (20mM). Lanes 5 (wt) and 6 (mut), experiment with ribozyme (1 pmol) and extra $MgCl_2$ (30mM). Lanes 7 (wt) and 8 (mut), experiment with extra ribozyme (2 pmol), extra MgCl (30mM).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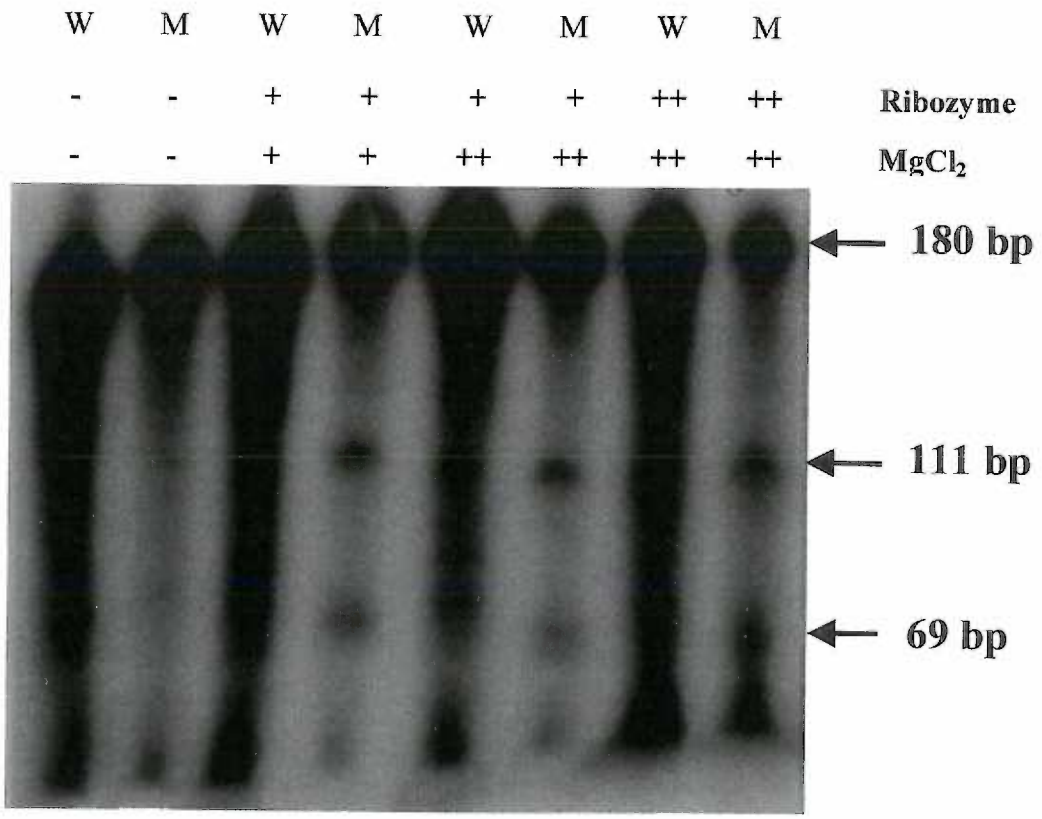
Target RNA 5'. . . GCCCAUGGGCCCUA UGGGACCCCGTGG . . .3'
Cleavage Site

A


W M W M W M W M
- - + + + + ++ ++ Ribozyme
- - + + ++ ++ ++ ++ MgCl2
180 bp
111 bp
69 bp

B

RESULTS

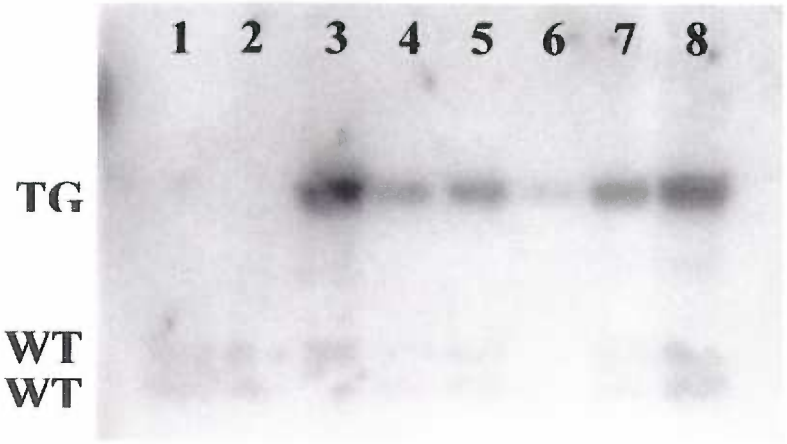
IN VITRO RIBOZYME CLEAVAGE RESULTS

Initial *in vitro* ribozyme cleavage experiments involved combining ribozymes with either the wild type or mutant target mRNA sequences. The wild type and mutant sequences were transcribed in the presence of ^{32}P-UTP and then were gel purified. The unlabeled ribozyme was then combined with wild type and mutant transcripts (180 bp) separately. Activity of the ribozyme is Mg^{2+} dependent. Therefore, the addition of $MgCl_2$ initiates the reaction while EDTA stops it. Ribozyme experiments were optimized by altering several variables: the ribozyme to target RNA ratio, $MgCl_2$ concentration, RNAse inhibitor, and incubation times and temperatures. When experimental reactions were conducted *in vitro*, ribozyme cleavage was specific for the mutant target (Figure 1-2B).

ANALYSIS OF TRANSGENIC MICE

Founder mice were identified by Southern blot methods after digestion of tail genomic DNA. Genotyping based on the presence or absence of the *NcoI* site identified one male and five female transgenic founders (Figure 1-3A). Quantitation showed that one male founder had 5 copies (lane 8), while the female founders ranged from 2 to 6 copies of the transgene (lanes 3-7). All founders appeared to be phenotypically normal, with the exception of one female founder (lane 6), which later developed a severe scoliosis at approximately four months of age and was not used in the study.

Figure 1-3. Genotype and phenotype of wild-type and transgenic mice. (A) Identification of transgenic founder mice. Tail genomic DNA from two wild-type mice (lanes 1-2) and from six founder mice (lanes 3-8) were digested with *NcoI* and analyzed by Southern blot. The 1168bp fragment is diagnostic of the transgene, whereas the 628bp and 540bp fragments represent the endogenous gene. (B) Newborn wild type (left) and transgenic (right) littermates. (C) Cleared skeletons of wild type (left) and transgenic (right) newborn mice. (D) Cleared forelimbs of wild type (top) and transgenic (bottom) newborn mice. (E) Cleared hind limbs of wild type (top) and transgenic (bottom) newborn mice. Alcian blue stains proteoglycan indicating cartilage, whereas alizarin red stains mineralized bone in C-E. There is marked shortening of bones in the skeletons of transgenic mice.



A

B

C

D

E

Transgenic progeny were obtained from the male (Figure 1-3A, lane 8) and one of the female founders (Figure 1-3A, lane 3). Transgenic offspring did not survive; they were severely dwarfed and died soon after birth from respiratory distress (Figure 1-3B). All surviving pups were wild type by Southern blot. Because of difficulties in obtaining transgenic newborns suitable for study, litters from the male founder were obtained by Cesarean section at 19.5 days post-coitus (dpc). Transgenic embryos had short limbs and trunk, small thorax, distended abdomen, short snout, protruding tongue and cleft palate (Figure 1-3B).

Cleared Skeletal Staining

Mouse skeletal phenotypes are often studied by staining cleared skeletons with alcian blue and alizarin red, which detect the presence of the glycosaminoglycan component of proteoglycans (cartilage) and calcified tissue (bone), respectively. Staining of the cleared 19.5 dpc transgenic skeletons showed the marked shortening of limb bones, ribs and vertebral column compared to wild type littermate embryos (Figures 1-3C, D and E). When compared to wild type cartilage tissues, transgenic cartilage did not stain well with alcian blue. Aside from smaller size, alizarin red S staining revealed that mineralization of primary and secondary ossification centers was delayed. This was confirmed radiographically, as well (data not shown).

Structural and Ultrastructural Analysis of Cartilages

The most striking difference between the transgenic and wild type littermates was the disorganization of the chondrocyte regions of the growth plates in developing transgenic bones (Figure 1-4A and B). The growth plate was particularly disordered in the hypertrophic zone where the usual columnar arrangement of cells was absent (Figure 1-4C and D). In addition, there was a marked reduction of chondrocytes in the proliferative zone in transgenic tissues.

Immunofluorescence for type II collagen showed decreased staining in the transgenic samples (Figure 1-5A and B). Aggrecan staining was decreased as well; however, because there was less collagen in the matrix, aggrecan may have been lost due to the histological processing (Figure 1-5C and D). Type X collagen localized to the hypertrophic zones displaying the disorganized features of transgenic tissue (Figure 1-5E and F).

Electron microscopy revealed a marked reduction of collagen fibrils in resting cartilage matrix from transgenic compared to wild-type mice. This reduction was observed in the pericellular matrix (Figure 1-6A and B) and in matrix between the cells (Figure 1-6C and D). Immunoelectron microscopy using antibodies specific for collagen types II (Figure 1-7A and B), IX (Figure 1-7C and D) and XI (Figure 1-7E and F) showed no difference between normal and transgenic samples, with exception of reduced number of fibrils.

Figure 1-4. Histological analysis of the growth plate cartilage stained with hematoxylin, eosin and alcian blue. Newborn wild type (A,C) and transgenic (B,D) proximal tibia growth plates are shown (A and B, magnified 100X; C and D, magnified 200X). There is a loss of normal columnar organization in transgenic samples.

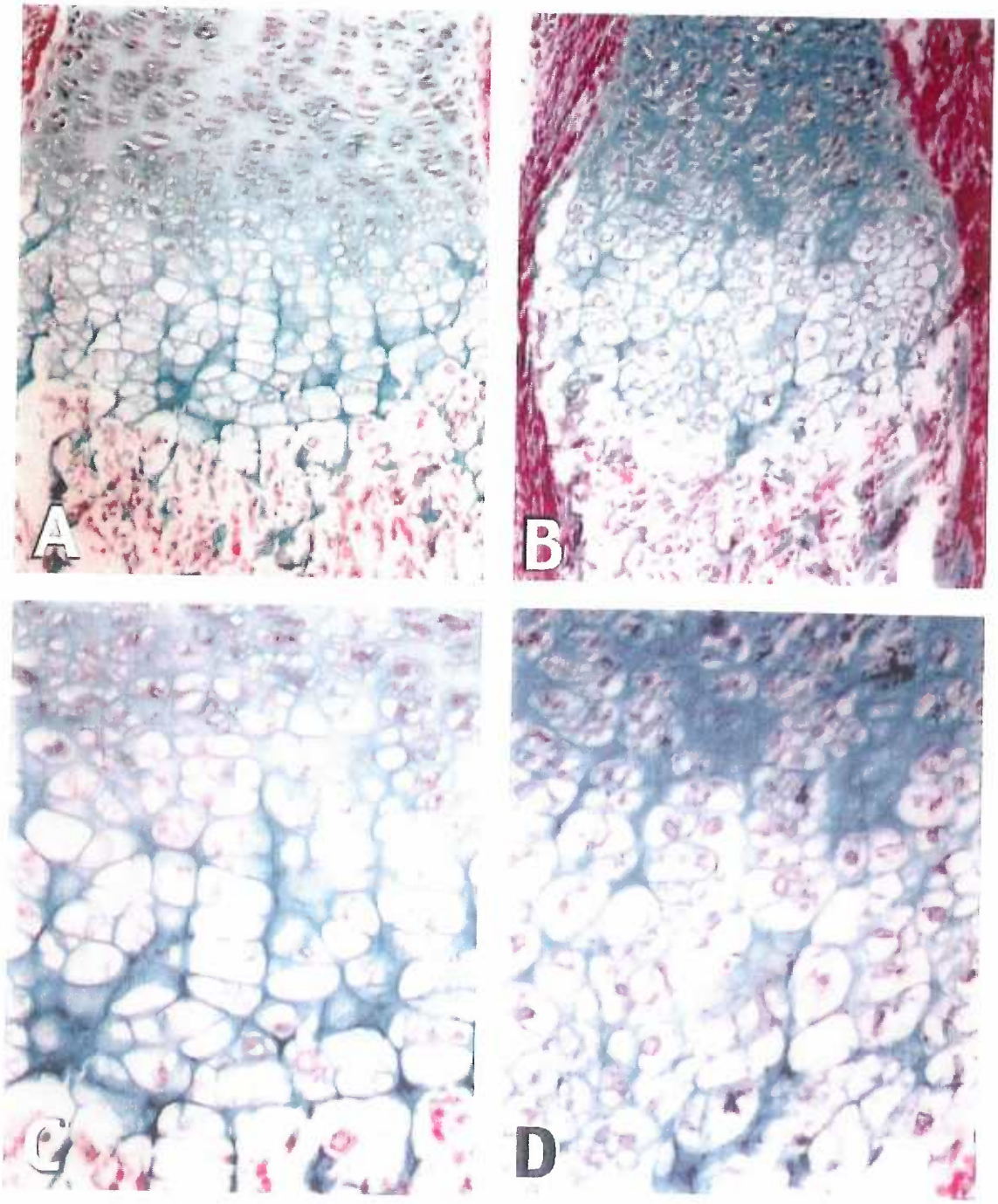
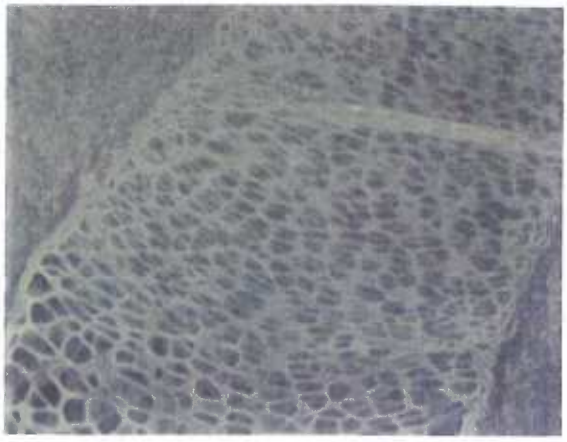

A
B
C
D

Figure 1-5. Immunohistochemistry of growth plate cartilage. Wild type (A-proximal tibia, C-distal tibia, E-distal tibia) and transgenic (B-distal ulna, D-distal tibia, F-distal tibia) tissues, from newborn mice, shown with antibodies against collagen type II (A,B), aggrecan (C,D) and collagen type X (E,F). The presence of collagen type II and aggrecan is decreased in transgenic samples (B, D).

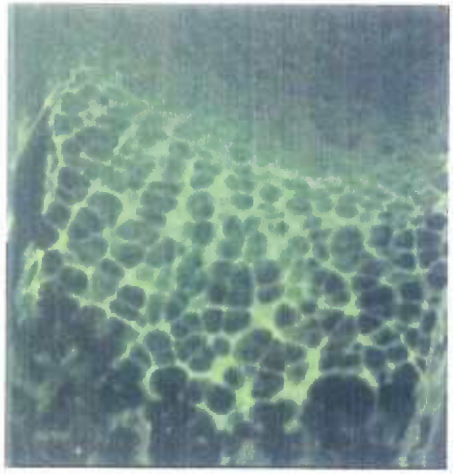
A

B

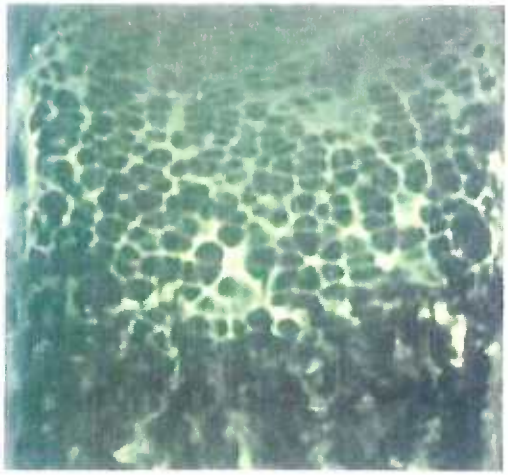
C



D



E



F

Figure 1-6. Electron microscopy of cells and matrix from cartilage of 16.5 dpc mice. Wild type (A) and transgenic humerus growth plate chondrocytes in the resting zone (magnified 12,000X). Normal (C) and transgenic (D) matrix surrounding chondrocytes in the resting zone (magnified 50,000X). There is a marked reduction of collagen fibrils in transgenic samples.

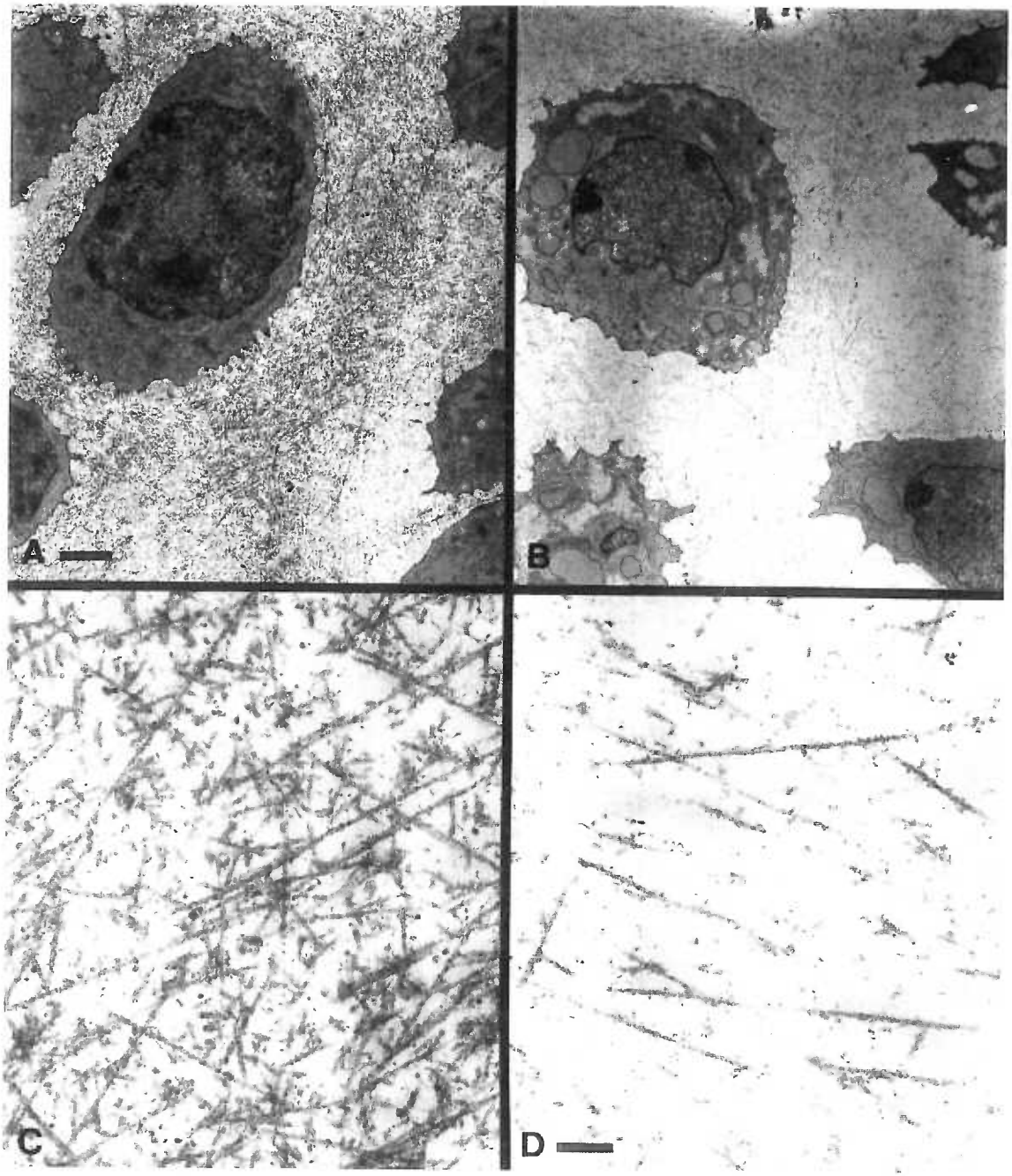
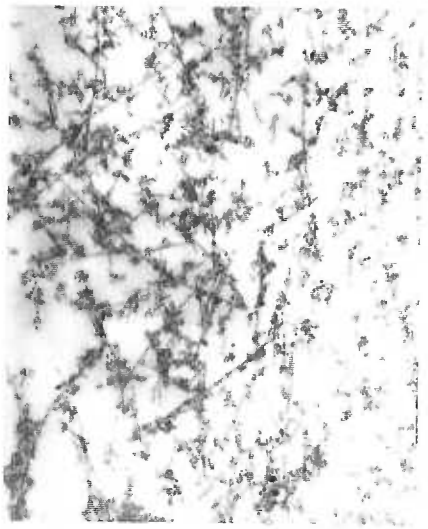
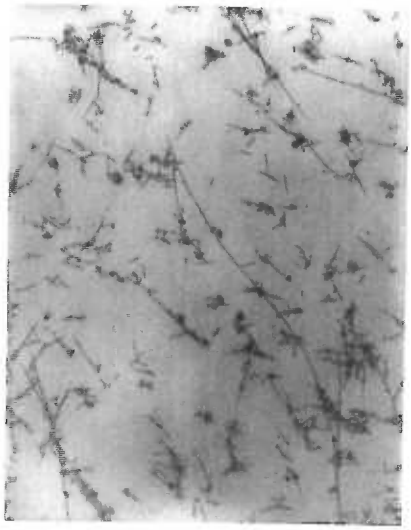

A
B
C
D

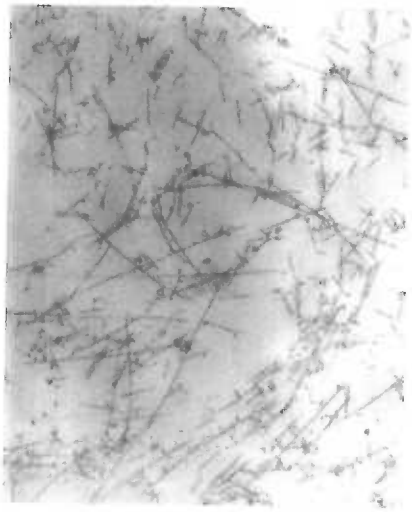
Figure 1-7. Immuno-electron microscopy of wild type and transgenic cartilage matrix. Fore limbs from wild type (A, C, E) and transgenic (B, D, F) 16.5 dpc embryos were analyzed using antibodies against collagen types II (A,B), IX (C,D) and XI (E,F). Immuno-gold labeling corresponding to collagen type II is decreased in the transgenic samples when compared to wild-type. A reduced number of fibrils were observed in all transgenic samples.



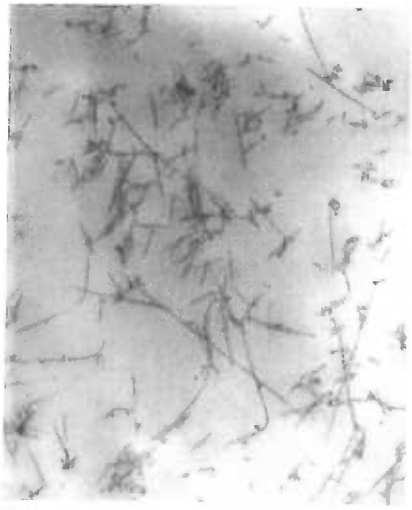
A



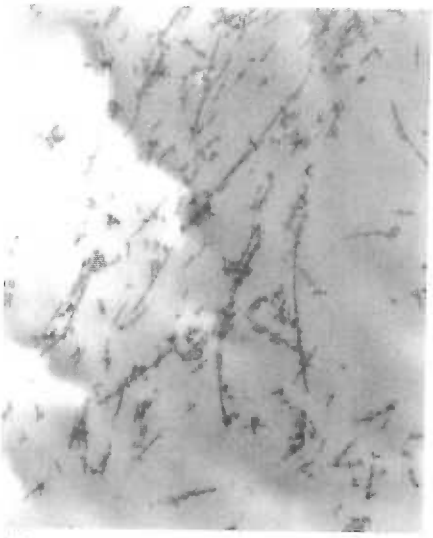
B



C



D



E

F

EXPRESSION OF MUTANT AND ENDOGENOUS Col2a1 TRANSCRIPTS

The amount of transgene and endogenous *Col2a1* mRNA was estimated in limb cartilages from the wild type and mutant littermates. Diagnostic cDNA fragments were generated by RT-PCR followed by digestion with *NcoI* or *SmaI* (Figure 1-8A and B) (Metsäranta et al., 1992). The relative densities of these fragments suggested that the mutant and endogenous *Col2a1* transcripts were present in equal amounts (Figure 1-8C and D).

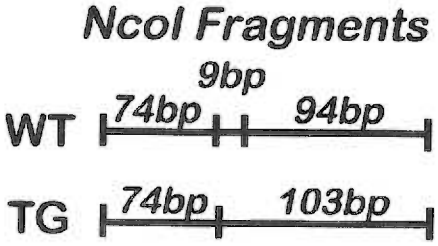
ANALYSIS OF TYPE II COLLAGEN SYNTHESIZED BY CELLS IN CULTURE

To test the fate of mutant procollagen chains, rat chondrosarcoma cells (RCS) were stably transfected with the R789C type II collagen construct used to generate the transgenic mouse strain. Equal numbers of transfected and untransfected RCS cells were cultured. Pepsin digests of medium and cell fractions were analyzed by western blot, using a type II collagen mouse monoclonal antibody. The cell fractions also contained the pericellular matrix. Type II collagen was detected in the cell fractions of both untransfected and transfected cells (Figure 1-9A, lanes 1, 3). However, protein was detected in the media fractions from only the untransfected cells (Figure 1-9A, lanes 2, 4). There was no evidence of dimers in the cell or medium fractions from the transfected cells (Figure 1-9A, lanes 3, 4).

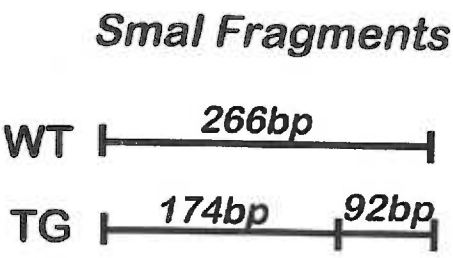
Untransfected and transfected cells were labeled with ^{35}S-cysteine for 24 hours in the presence of ascorbic acid. The label was detected in high molecular weight bands that were not identified in cell/pericellular and medium fractions from both nontransfected and transfected cells (Figure 1-9B, lanes 2-5). A band was detected in the

cell fraction from transfected cells, but not untransfected cells (Figure 1-9B, lane 4). This band corresponded in mobility to the α1 band that labeled with ^{3}H-proline (Figure 1-9B, lane 1, arrow) and stained with antibody to type II collagen (Figure 1-9A, lane 3). This experiment was repeated by separating the cells from the pericellular matrix. It was determined that the mutant chain is retained intracellularly.

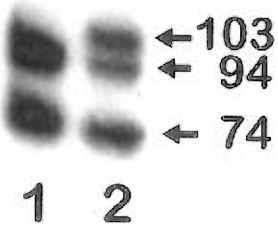
Figure 1-8. Transgene expression. Predicted (A, B) and observed (C, D) sizes of wild type and transgene *Col2a1* cDNA fragments following RT-PCR of limb RNA and digestion with *NcoI* (A, C) or *SmaI* (B, D). Transgenic samples are shown in lane 2 in both C and D. Densitometric quantification showed that the transgene and endogenous genes were expressed at similar levels.


NcoI Fragments
9bp
WT
74bp
94bp
TG
74bp
103bp

A


SmaI Fragments
WT
266bp
TG
174bp
92bp

B

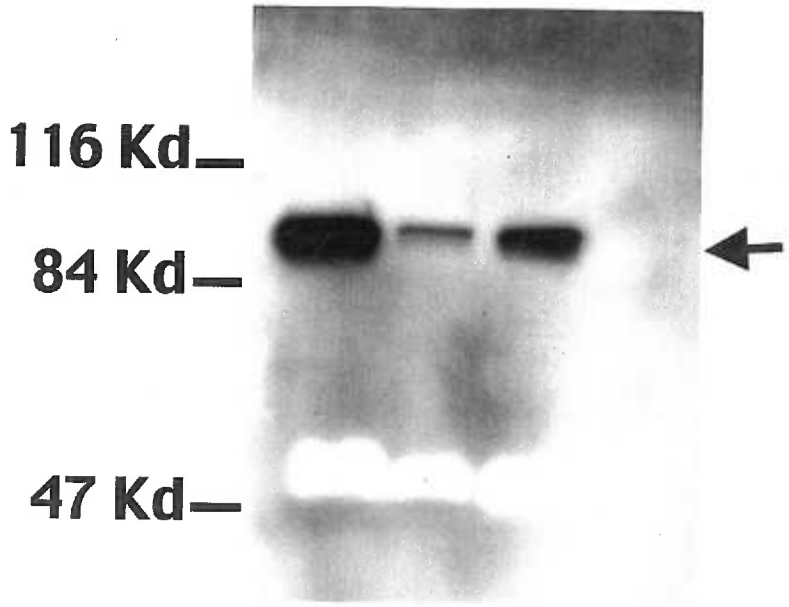

103
94
74
1
2

C

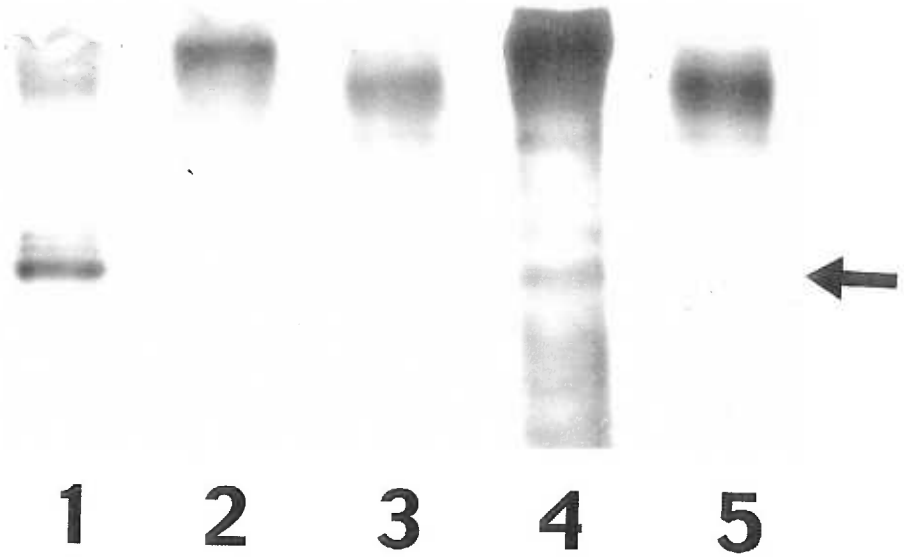

266
174
92
1
2

D

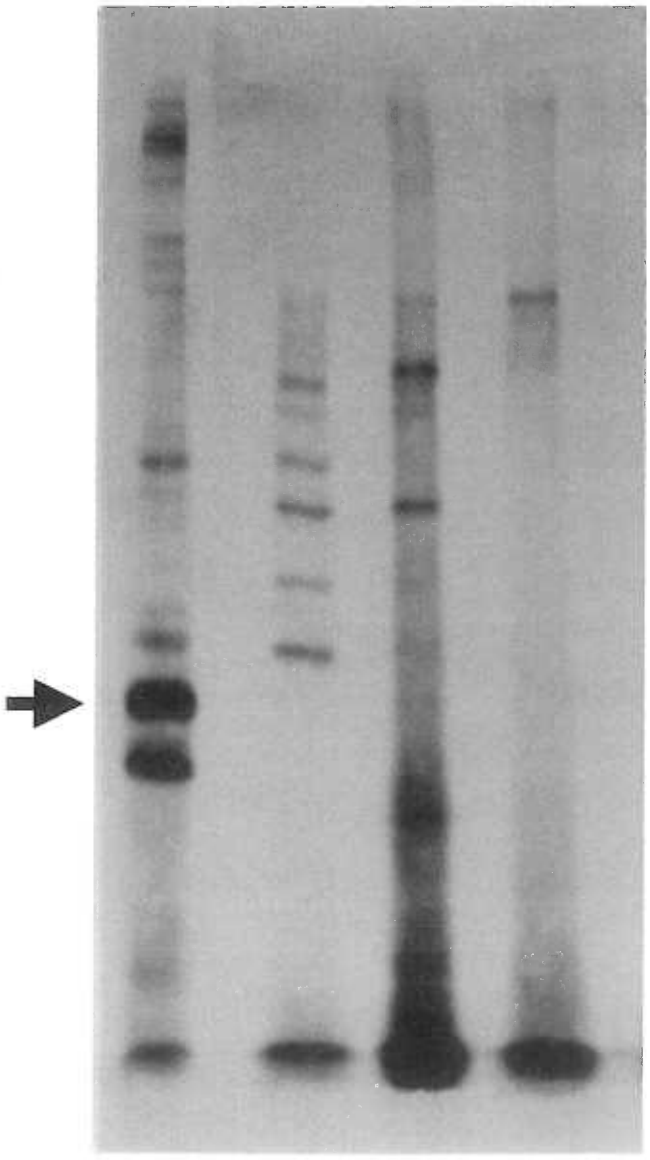
Figure 1-9. Protein analysis of rat chondrosarcoma cells transfected with the transgene. (A) Western analysis of pepsin-digested extracts from untransfected (lanes 1-2) and transgene transfected (lanes 3-4) cells, using a type II collagen antibody. Bands corresponding to α1(II) (arrow) are found in the cell/pericellular matrix fractions (lanes 1, 3) and to a lesser extent in the media fraction of the untransfected cells (lane 2). Type II collagen was almost absent in the transfected cell media (lane 4). (B) Untransfected cells (lanes 2-3)) and transfected cells (lanes 4-5) were labeled with ^{35}S-cysteine. The pepsin-digested proteins were run on a nonreduced polyacrylamide gel. Only the transfected cell fraction (lane 3) contained a cysteine labeled band corresponding to the position of the αI(II) in the ^{3}H-proline labeled control (lane 1). (C) Transfected cells labeled with ^{35}S-cysteine were again studied by separating the fractions, pericellular matrix (lane 2), cell lysate (lane 3) and media (lane 4), to determine if the mutant chain is secreted into the pericellular matrix. Chicken tendon fibroblasts were labeled with ^{14}C-proline to be used as a positive control (lane 1). The mutant chain (corresponding to the arrow) is not secreted from the cells.



A



B



C

DISCUSSION

Cartilage targeting of the Y-position type II collagen mutation, R789C, produced transgenic mice with a marked decrease of collagen fibrils. Of the several reported strains of mice mutant for *Col2a1*, the transgenic mice studied here most closely resemble the *Col2a1* null mice described by Li et al (Li et al., 1995). Both showed severe delay in skeletal development, profound shortening of limbs, short snout and cleft palate. Histologically, the epiphyseal growth plate had a reduced amount of matrix and lacked the usual cellular organization. The greatest similarity is the marked reduction in collagen fibrils in cartilage matrix. This observation suggests that phenotypic severity depends on the extent to which cartilage collagen fibril formation is disturbed or decreased.

One of my goals was to experimentally model a form of human SED in which the R789C mutation had been observed (Chan et al., 1993). While a chondrodysplastic phenotype was produced, several differences were noted between the human and mouse conditions. A major difference was severity. The transgenic mice died at birth and their skeleton was more severely affected than that of the human. This is probably due to lower amount of cartilage collagen fibrils in the mouse, although there may be subtle differences in the role of type II collagen in endochondral ossification in the two species. Another difference is gene dosage. The human disorder results from the expression of two alleles: one mutant and one wild type *COL2A1* allele. Whereas, the mouse phenotype results from expression of several copies of the mutant *Col2a1* transgene superimposed on that of two endogenous wild type *Col2a1* alleles. More transcripts are produced in the latter situation. However, the ratio of wild type and mutant transcripts in

our mice appears to be 1:1, the same as in the human disorder. Of interest is that only one third of the α1(II) chains isolated from a patient harboring the R789C mutation were mutant (Chan et al., 1993).

Another goal of this study was to use a ribozyme based therapy to inhibit translation of the mutant α chain. The ribozyme was constructed to target a cleavage site present in the transgene, but absent in the endogenous *Col2a1* genes. The ribozyme specifically cleaved the targeted RNA transcript *in vitro*. However, it was never able to cleave all the transcripts presented. Because the cleavage efficiency was lower than desired and the R789C transgenic phenotype was more severe than expected, experiments to test the ribozyme strategy in mice were discontinued.

The presence of disulfide-linked α1(II) dimers was reported in cartilage extracts from one case of human SED associated with the R789C mutation (Chan et al., 1993). We were unable to analyze mouse cartilage tissues with similar methods due to their very small size. Thus, direct comparisons of the human and mouse findings were not possible in this regard. However, by western blot analysis, dimers were not observed in the untransfected or transgene transfected RCS cells. It is not clear if the discrepancy in dimer formation, which may account for some phenotypic differences between humans and mice, reflects biological differences between the two species or differences in how analyses were done.

The observations regarding dimer formation are consistent with those of Fertala et al who studied another Y-position *COL2A1* mutation, R519C (Fertala et al., 1997). From analysis of recombinant proteins containing the R519C mutation, they concluded that the mutation did not generate intramolecular disulfide bonds as had been demonstrated for

cysteine substitutions at the Gly-position. They suggested from computer modeling experiments that this is due to the relatively greater distance between cysteine residues occupying the Y-position compared to the Gly-position during collagen helix formation. In collaboration with James Bann and Hans Peter Bächinger, our molecular modeling results suggested similarly that helix assembly would not be adversely affected by this mutation (Appendix 1). However, we did not compare Y- and Gly-position distances for the R789C mutation.

The western blot analysis showed a reduced amount of collagen type II in the cell/pericellular matrix fraction and very little detected in the media fraction of transfected cells. To investigate if the procollagen molecules in the cell/pericellular fraction contained mutant α chains, we biosynthetically labeled the cells with ^{35}S-cysteine. Only the mutant type II collagen chains would contain a cysteine within the pepsin-resistant triple helical domain. It appears from the data that the mutant chains are incorporated into procollagen molecules in the transfected cell/pericellular matrix sample and are not present in the media sample.

Further experiments confirmed that collagen molecules, which are composed of mutant chains, are retained intercellularly. Fibril assembly is a post-secretion event that occurs after the processing of the procollagen molecules to the mature species. The data suggests that mutant molecules are not secreted and, therefore, are unavailable to contribute to fibril formation. This circumstance would eventually result in a decreased amount of collagen fibrils. However, it is not understood how many mutant chains is required to disturb the function of the procollagen molecule by intercellular retention.

Our findings of greatly reduced cartilage fibrils can best be explained by a dominant negative mechanism, where collagen molecules containing mutant and wild type chains fail to assemble into fibrils. Others have also demonstrated disturbances in fibril assembly and morphology when recombinant collagen chains containing the Y-position R519C mutation were present (Adachi et al., 1999; Fertala et al., 1997). The fibrils were loosely packed and some lacked a D-periodic banding pattern. Molecules comprised of mutant monomers did not form fibrils under conditions which promoted assembly of fibrils from molecules containing only wild type monomers. Similarly, the results presented here suggest that mutant procollagen α chains are incorporated into pepsin-resistant type II collagen molecules which fail to assemble into fibrils, the deficiency of which accounts for the skeletal phenotype.

CHAPTER 2

The Analysis of Transgenic Mice with an Assembly Mutation in Cartilage Oligomeric Matrix Protein

BACKGROUND

Cartilage oligomeric matrix protein (COMP) is a non-collagenous glycoprotein. It is found abundantly in cartilage, ligament, tendon and synovium (DiCesare et al., 1994; Fife and Brandt, 1984; Hecht et al., 1998; Hummel et al., 1998; Maddox et al., 1997) (Figure 2-1A). COMP is a member of the thrombospondin family, sharing significant homology and potential functions with them. Mutations within the COMP gene have been found to cause two types of dominantly inherited chondrodysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED). These disorders are characterized by moderate to severe short stature and early onset osteoarthritis. The biological function of COMP is not understood; however, because its mutations cause skeletal abnormalities, COMP is thought to play a crucial role in bone development and growth.

THROMBOSPONDIN GENE FAMILY

COMP is a member of the thrombospondin (TSP) gene family. The members, TSP1,-2,-3,-4 and COMP, share many structural features and are thought to perform similar functions. They are all extracellular matrix proteins and modulate a variety of cell behaviors, such as adhesion, migration and proliferation. There is evidence to suggest that TSP1 has roles in hemostasis, angiogenesis, wound healing, neurite outgrowth and metastatic tumor progression (Grant et al., 1998; Mosher, 1990; Osterhaut et al., 1992; Tolsma et al., 1993; Watkins et al., 1990). The TSP gene family members feature 3 or 5 monomers connected by an amino-terminal coiled-coil domain (Figure 2-

1C). All TSPs have monomers which contain the characteristic epidermal growth factor (EGF)-like domains, calcium binding repeats and globular carboxyl-terminal ends. To assess the potential biological roles of COMP, it is important to review what is known about related proteins.

TSP1 was the first to be described and is the best characterized today (Baenziger et al., 1971; Lawler et al., 1978). It is a trimeric protein that is synthesized at high levels by many different cell types, including developing heart, lung, liver, brain, kidney, bone and skeletal muscle. It is observed in developing mouse cartilage after 16 dpc, although its role here is not understood (Iruela-Arispe et al., 1993). The three monomers are assembled by a coiled-coil domain, followed by two cysteine residues, which participate in interchain disulfide bonding. A procollagen domain (homologous to the amino terminus of human collagen I), three properdin-like repeats and three EGF-like repeats are located adjacent to the coiled-coil domain (Vuorio and de Crombrugghe, 1990) (Figure 2-1C). Properdin is a molecule found to play a role in immunity and homeostasis (Maves and Weiler, 1993). The homologous region of properdin and TSP1 contains binding sites for collagen type V, laminin and fibronectin. Towards the carboxyl-terminal end, there are eight calcium binding repeats (also referred to as calmodulin-like repeats) and a globular end.

TSP1 is incorporated into the extracellular matrix where it has the ability to interact with other matrix proteins, cell surface receptors and cations. In addition to calcium, it binds heparan sulfate proteoglycans, collagen type V, plasminogen, fibrinogen, fibronectin, urokinase, the $\alpha\upsilon\beta3$, $\alpha3\beta1$, $\alpha4\beta1$ and $\alpha5\beta1$ integrins, the integrin associated protein and a cell surface protein, CD36 (Adams and Lawler, 1993; Asch et

al., 1993; DeFreitas et al., 1995; Gao et al., 1996; Mumby et al., 1984; Tsao and Mousa, 1995; Yabkowitz et al., 1993). TSP1 also has the ability to activate transforming growth factor (TGF) β1 (Crawford et al., 1998; Schultz-Cherry et al., 1995). TSP1 interacts with the N-terminal region of latency-associated protein, which is bound to TGF-β1. A conformational change then alters the immunoreactivity of TGF-β1 making it accessible to its receptor. Through various interactions, TSP1 is involved in platelet aggregation, the attachment and spreading of various cell types, promoting motility of human neutrophils, promoting tumor metastasis, inhibiting angiogenesis and smooth muscle cell proliferation (Tuszynski, 1993).

To further investigate TSP1 function, the mouse gene was disrupted by homologous recombination (Lawler et al., 1998). Most of the TSP1-deficient mice were viable, fertile and displayed very subtle developmental abnormalities. It is surprising that no major abnormalities were observed in brain, heart, kidney, spleen, stomach, intestines, aorta or liver. However, in the TSP1-deficient mice, the lungs showed characteristics of acute pneumonia. At one to four months of age, the TSP1-deficient mice had increased inflammatory cell infiltrates and epithelial cell hyperplasia in the lungs. Their results suggest that TSP1 is involved in normal lung homeostasis.

It is interesting to note that the TSP1 null mouse and the TGF-β1 null mouse have striking similarities (Crawford et al., 1998; Lawler et al., 1998). TGF-β1 is a cytokine that is involved in cell growth, differentiation and immune modulation (Lawrence, 1996). TGF-β1 is primarily regulated extracellularly where the secreted latent form must be proteolytically activated. TSP1 plays a primary role in the conversion of TGF-β1 from latent to active forms (Crawford et al., 1998; Murphy-Ullrich et al., 1993; Schultz-Cherry

and Murphy-Ullrich, 1993). Because the TGF-β1 null mice did not survive more than four weeks, due to an autoimmune-based inflammation of several organs, both mouse lines were compared at 2 ½ weeks of age. When compared to TSP1-deficient mice, the TGF-β1 null mice had very similar lung pathologies, including vessel abnormalities, thickened bronchiolar arteries, capillary leakage and alveolar hemorrhage. Although TSP1 is not highly expressed in the pancreas, abnormal pathologies were observed in both null mouse lines, such as increased islet cells and moderate to severe inflammation. These same phenotypes could be induced in wild-type mice, when given a systemic treatment of a peptide that blocked the activation of TGF-β1 by TSP1 (Crawford et al., 1998).

The structure of TSP2 shows considerable resemblance to TSP1. They are both homotrimers, connected by a coiled-coil assembly domain. Following the coiled-coil domain, two cysteines are positioned for interchain disulfide linkage. TSP1 and TSP2 each have a procollagen homology domain and properdin repeats at the amino-terminal end (Figure 2-1). Having comparable structure suggests that they probably interact with the same cell surface receptors (Chen et al., 1994; Chen et al., 1996). Like all other family members, TSP2 has EGF-like repeats, a series of calcium binding domains and a globular carboxyl-terminal end. The significant homology between TSP1 and TSP2 allows for the assembly of heterotrimers composed of both polypeptides (O'Rourke et al., 1992).

Although TSP1 and TSP2 have almost identical coding sequences with similar exon/intron boundaries, the DNA sequence corresponding to the promoter region, is very different. Therefore, TSP2 responds differently to growth factors or serum and has

contrasting expression patterns in the developing embryo and adult mouse, when compared to TSP1 (Bornstein, 1992). TSP2 is expressed primarily in connective tissues, such as dermis, tendon, ligament, cartilage, smooth muscle, endothelial cells, perichondrium and pericardium (Iruela-Arispe et al., 1993; Kyriakides et al., 1998). In adult mouse tissues, TSP2 was also detected in brain, testis and adrenal glands. TSP2 has been localized to areas of chondrogenesis, osteogenesis and vasculogenesis in 15 to 18 dpc mouse embryos (Kyriakides et al., 1998).

To explore a functional role for TSP2, the mouse Thbs2 gene was disrupted by homologous recombination (Kyriakides et al., 1998; Kyriakides et al., 1998). The TSP2-deficient mice were normal in appearance and fertile. However, these animals had abnormalities in various connective tissues, including blood vessels, bone, skin and tendon. When genotyping, the bleeding time from tail snips was abnormally long in mutant mice. This probably resulted from an increase in the number of blood vessels. On average, TSP2-deficient mice were reported to have twice as many small blood vessels, in skin and other tissues, when compared to wild-type littermates.

The TSP2-deficient mice had additional connective tissue abnormalities. Their skin had increased laxity and fragility. Mutant skin was very stretchy and easily torn, as observed from conflicts between littermates. When injured, mutant mice required additional time to heal. Furthermore, cultured mutant skin fibroblasts were unable to attach to substrates of many different extracellular matrix proteins. TSP2 null mice had extreme flexibility of their ligaments and tendons allowing their tails to be tied in a knot. In addition, the TSP2-deficient mice did feature increased cortical thickness and density of long bones, which may occur in postnatal development. However, in the absence of

TSP2, there did not seem to be an effect on chondrogenesis. Sections of developing cartilage appeared histologically normal (Kyriakides et al., 1998).

A closer investigation of skin and tendon tissues from TSP2-deficient mice revealed collagen fibrils that were disordered and enlarged. In addition, fibrils had irregular contours when seen under an electron microscope. Initially, the data suggested that TSP2 may have a role in collagen fibrillogenesis. However, in further studies, collagenous matrices from normal mouse tissues did not show any immuno-reactivity towards TSP2 (Kyriakides et al., 1998). Currently, the role of TSP2 in collagen fibrillogenesis is unclear.

There is very limited data for other thrombospondin family members and their functions remain unknown. TSP3, -4 and COMP are all pentameric proteins and have highly homologous sequences to each other. They have five identical monomers that are connected by a coiled-coil domain. Unlike TSP1 and TSP2, their monomers do not have a procollagen domain or properdin repeats. Because many of the proposed functions of TSP1 and TSP2, including angiogenic activity and activation of TGF-β1, involve these amino-terminal domains, the roles of these other TSP members are thought to be quite different. However, they do have four EGF-like domains, eight calcium binding domains and a carboxyl-terminal end (Bornstein and Sage, 1994).

The pentameric TSPs are differentially expressed as well. TSP3 is primarily expressed in developing lung, with substantial levels in bone, skin, skeletal muscle and heart (Vos et al., 1992). There is virtually nothing known about its biological roles. TSP4 has recently been isolated from tendon, neurons, cardiac and skeletal muscle (Arber and Caroni, 1995; Hauser et al., 1995; Lawler et al., 1993). It is thought to have a role in

myoblast adhesion and promoting neurite outgrowth (Adams and Lawler, 1994; Arber and Caroni, 1995). As mentioned earlier, COMP is located in cartilage, ligament, tendon and synovium (DiCesare et al., 1994; Fife and Brandt, 1984; Hecht et al., 1998; Hummel et al., 1998; Maddox et al., 1997). COMP mutations have been found to cause two autosomal dominant forms of short limbed dwarfism, pseudoachondroplasia and multiple epiphyseal dysplasia (Briggs et al., 1995; Cohn et al., 1996; Hecht et al., 1995). Due to the effects of these mutations on human bone formation, COMP is thought to have an important role in skeletal development.

CARTILAGE OLIGOMERIC MATRIX PROTEIN

COMP is a pentameric structure composed of five identical monomers (Figure 2-1A). Each monomer has 755 amino acids and a molecular mass of 100-120 kDa, depending on the degree of glycosylation (Mörgelin et al., 1992; Oldberg et al., 1992). The complete pentameric protein is approximately 524 kDa. COMP has a bouquet-like shape with each arm containing a terminal globular domain, a flexible strand, and a coiled-coil domain, where the five chains are joined together (Mörgelin et al., 1992).

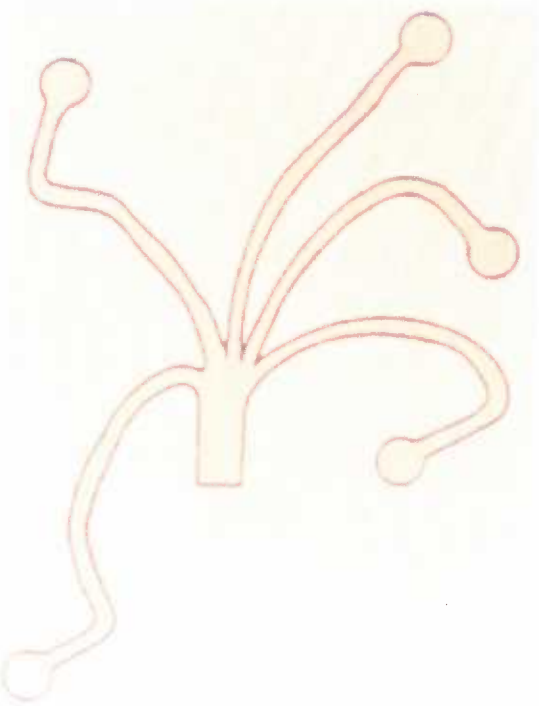
A coiled-coil structure is commonly used to facilitate assembly of larger, more complex proteins. The amino acid sequence of a coiled-coil domain is highly conserved (Engel, 1991). The α-helical bundles formed can result from a single chain folding back on itself or individual chains in parallel or anti-parallel orientation. The monomers of COMP are joined together by a five-stranded parallel α-helical bundle (Efimov et al., 1996; Malashkevich et al., 1996). The coiled-coil domain of COMP is very similar to other pentameric TSPs, but much shorter than TSP1 and TSP2. The amino acid sequence

is composed of heptad repeats $(a\text{-}g)_n$, with predominantly hydrophobic residues in the a and d positions and polar residues elsewhere. Two closely spaced cysteine residues, Cys-68 and Cys-71, are located following the heptad repeat sequence. The five chains can be covalently linked by the formation of interchain disulfide bonds. As with other α-helical coiled-coil domains, the coiled-coil domain of COMP is stabilized by hydrophobic interactions, disulfide bonds and salt bridges (Kajava, 1996).

COMP structure is very similar to TSP1 towards the carboxyl-terminal end; however, the amino-terminal end is quite different (Figure 2-1C). COMP does not have procollagen or properdin-like domains. However, following the amino-terminal assembly domain, COMP has four EGF-like repeats, eight calcium binding domains and a globular carboxyl-terminal end. The presence of EGF-like repeats gives COMP some homology to many cell surface and secreted proteins. The calcium binding domains are very rich in aspartic acid residues, with each repeat containing at least one sequence similar to the EF-hand, a calcium binding motif of calmodulin. Upon binding to calcium, TSP1 is thought to undergo conformational changes, which may alter it's adhesive ability (Misenheimer and Mosher, 1995; Ugarova et al., 1995). COMP is thought to respond in a similar manner. The globular carboxyl-terminal end has been suggested to be an attachment site to other cartilage matrix proteins (Rosenberg et al., 1998).

COMP is found in all cartilages, including epiphyseal, articular, nasal and tracheal cartilage, as well as in the vitreous of the eye (Fife, 1988; Hedbom et al., 1992; Mörgelin et al., 1992; Nguyen and Fife, 1986). It appears to be most abundant in adult articular cartilage, where it is found in the matrix between the chondrocytes (DiCesare et al., 1994). However, in the developing growth plate, COMP is mainly located in the

Figure 2-1. COMP pentameric and monomeric structure. (A) A suggested model of COMP (Mörgelin et al., 1992). (B) COMP structure viewed by glycerol spraying/rotary shadowing electron microscopy (Mörgelin et al., 1992). Protein samples were obtained from Swarm rat chondrosarcoma tissue. (C) COMP, TSP3 and TSP4 monomeric subunit structure compared to that of TSP1 and TSP2.



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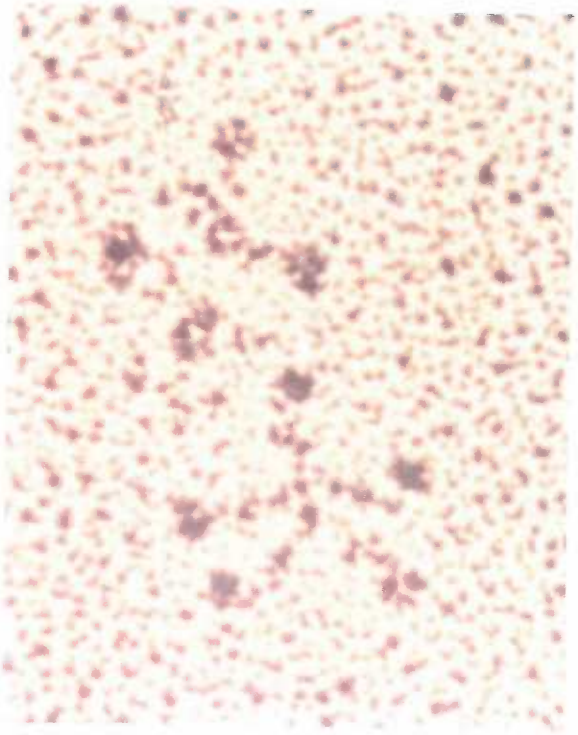


B


Thrombospondin 1 and 2
NH2
COOH
Coiled-Coil
Procollagen
Properdin
EGF-like repeats
Calcium-binding repeats
Globular end


Thrombospondin 3, 4 and COMP
Coiled-Coil
EGF-like repeats
Calcium-binding repeats
Globular end

C

proliferative zone, where it surrounds the chondrocytes in a pericellular distribution (Ekman et al., 1997; Shen et al., 1994). Zaia, et al has reported that there are glycosylation differences in adult and fetal COMP (Zaia et al., 1997). These changes may enable COMP to interact with various matrix or cell surface molecules, depending on the age and type of tissue.

Being a matrix protein, COMP has been shown to interact with other matrix molecules. COMP binds triple helical collagen in a zinc (or nickel)-dependent fashion (Rosenberg et al., 1998). The presence of calcium, magnesium or manganese does not promote binding to collagen. In fact, calcium partially inhibited the COMP-collagen interaction (Rosenberg et al., 1998). It is important to note that resting cartilage in the growth plate and articular cartilage have different calcium-zinc ratios (Rizzo et al., 1995; Vittur et al., 1992). This may explain COMP's ability to have variable types of interactions with a protein depending on its location. By co-immunoprecititation, COMP has also been shown to be associated with TSP-4, either directly, indirectly through a third protein or by forming heteropentamers (Hecht et al., 1998).

Pseudoachondroplasia and Multiple Epiphyseal Dysplasia

The human COMP gene has been localized to chromosome 19p12-13.1 and it's cDNA has been cloned (Newton et al., 1994). COMP is composed of 19 exons. Exons 1-3 are unique to COMP, while exons 4-19 share significant sequence homology to the other TSP genes, particularly TSP3 and TSP4 (Bornstein et al., 1993; Li et al., 1996; Newton et al., 1994). After linkage between pseudoachondroplasia and several cartilage specific genes had been excluded, two large families with pseudoachondroplasia were

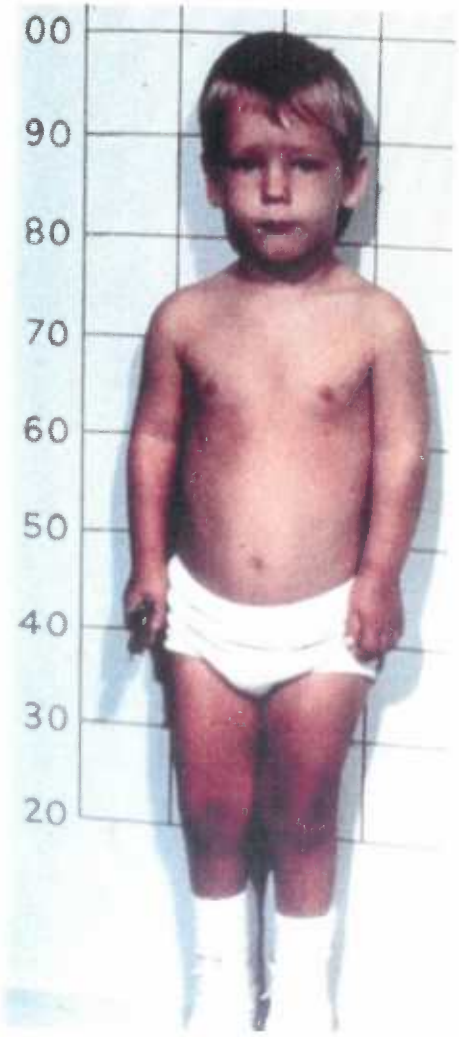
genetically linked to chromosome 19 and then later to the COMP gene locus (Briggs et al., 1995; Briggs et al., 1993; Hecht et al., 1993; Hecht et al., 1995).

Mutations within the COMP gene cause two dominantly inherited chondrodysplasias, pseudoachondroplasia (PSACH) and multiple epiphyseal dysplasia (MED) (Ballo et al., 1997; Cohn et al., 1996; Deere et al., 1998; Délot et al., 1999; Ikegawa et al., 1998; Maddox et al., 1997; Susic et al., 1997). PSACH is characterized by moderate to severe short stature, with adult heights ranging from 3'5" to 4'3" (Figure 2-2A) (Horton et al., 1982). PSACH patients have short limbs, joint laxity and normal craniofacial development (Wynne-Davies et al., 1986). Patients are often described as normal at birth, then develop a waddling gait that becomes evident at 6 months to 4 years of age. Through radiographs, PSACH is characterized by abnormal epiphyses and metaphyses of the hands, long bones and hips, often with anterior beaking of the vertebrae (Maroteaux et al., 1980). In childhood, surgical procedures are required to correct lower extremities from bowing. Whereas, hip replacements are very common upon adulthood. PSACH patients often have early onset osteoarthritis, and scoliosis may cause additional complications.

MED is often much less severe than PSACH. MED patients typically have normal to slightly short stature with osteoarthritis that appears in late childhood (Figure 2-2B). Pain and stiffness in joints, particularly in the hips, is common. MED patients seldom have spinal symptoms. Radiographically, the epiphyses are quite irregular and show very poor ossification. MED is divided into the more severe Fairbank type and the milder Ribbing type (Fairbank, 1947; Ribbing, 1937). COMP mutations have been found to cause both types of MED (Ballo et al., 1997; Briggs et al., 1995). In addition, collagen

Figure 2-2. PSACH and MED patient phenotypes. (A) PSACH patient, 10 years of age, is shown with disproportionate short stature and bowed legs (Maddox et al., 1997). (B) MED patient, 6 years of age, is shown with short stature. (C) Immunofluorescence using human anti-COMP antibodies show that COMP is retained in PSACH chondrocytes (Maddox et al., 1997). In normal human cartilage, COMP is localized extracellularly. (D) Electron micrographs show characteristic lamellar patterns of the rough endoplasmic reticulum in chondrocytes associated with these disorders (Maddox et al., 1997).

A



B

C

D

type IX mutations, in both the α2 and α3 chains, were recently reported to cause MED (Briggs et al., 1994; Paassilta et al., 1999). The mutations were mapped to chromosome 1p32 (COL9A2) and chromosome 20 (COL9A3) with no evidence for linkage to other candidate genes, COMP or COL9A1. There is evidence that COMP and type IX collagen interact; however, a direct interaction has not been shown (Hecht et al., 1998).

Cartilage samples of PSACH and MED patients show an accumulation of COMP, and other matrix proteins, in the chondrocyte rough endoplasmic reticulum (rER) (Maddox et al., 1997; Maynard et al., 1972; Stanescu et al., 1982). Microscopy using antibodies to aggrecan show a distinct lamellar pattern of material within the rER. This histological phenotype has been associated with these disorders for many years, without an explanation as to why these fingerprint patterns develop (Figure 2-2C). Originally, this suggested that there is a problem in proteoglycan synthesis or degradation. However, genetic linkage of PSACH or MED to aggrecan has been excluded, and the effects on aggrecan are thought to be secondary to the COMP mutation (Finkelstein et al., 1991). Based on immunofluorescence, type IX collagen and link protein were also found to accumulate within the rER (Maddox et al., 1997; Stanescu et al., 1982). This retention of material may suggest a disruption in protein biosynthesis or degradation.

Most COMP mutations are located within the calcium binding repeat domains. Several types of mutations have been documented: single base substitutions, an expansion of trinucleotide repeats and small deletions. The result of most mutations involves the replacement of an aspartic acid residue which is suspected to disturb calcium binding and, in turn, alter COMP structure (Klee et al., 1980). In the absence of calcium, the structure of TSP1 changes to a form which is unable to adhere to platelets (Dixit et

al., 1986; Lawler et al., 1985). The transition of TSP1 from its adhesive to its non-adhesive conformation is regulated by bound calcium ions. Due to the shared sequence homology to TSP1, the structure and adhesive properties of COMP are probably calcium dependent as well. The MED mutations are thought to have a more subtle effect on COMP structure when compared to the PSACH mutations, perhaps explaining the difference in phenotype severity.

How these COMP mutations prevent proper bone growth is not completely understood. Because the mutations are found towards the carboxyl-terminal end, the assembly of COMP pentamers, which occurs at the amino-terminal end, is expected to occur normally. However, due to mutations within the calcium binding domains, it is likely that COMP structure is altered. The PSACH and MED patient chondrocytes feature large rER compartments, where COMP molecules, and other matrix proteins, are retained. This could lead to a deficiency of matrix molecules which are required for correct bone development (Maddox et al., 1997). Another hypothesis is that due to the consequences of a block in protein processing, the chondrocytes are unable to perform their functions and undergo premature apoptosis (Hecht et al., 1998). The resulting bone phenotype is probably due to a combination of having fewer cells, which in turn, are secreting less matrix.

Osteoarthritis

Before COMP mutations were found to cause PSACH and MED, COMP was studied in reference to osteoarthritis (OA) (Dieppe, 1995; Hutton, 1995). OA is the most common joint disease which causes patients great disability and discomfort. Developing

OA is a gradual process, where initial joint damage may be followed by further cartilage degradation. Usually by the time a radiological diagnosis is made, the established disease is present and there is significant deterioration of the joint and cartilage loss. Understanding the molecular events that initiate this disease and those that occur upon cartilage degeneration are crucial for early diagnosis, for monitoring the stages of disease progression and for developing new approaches to treatment. Identifying serum markers for cartilage degradation has become of interest for diagnostic use as well as for assessing the response to therapeutic treatments.

COMP is found abundantly in the matrix of adult articular cartilage and it's gene expression may be enhanced by high mechanical load (Smith et al., 1997; Wong et al., 1999). COMP levels, in serum and synovial fluid, have been studied as a potential diagnostic or prognostic marker for detecting OA. However, within synovial fluid samples, COMP fragments could originate from degrading cartilage or from the synovial cells. Therefore, using the quantitation of COMP fragments from synovial fluid as a marker for cartilage degradation has not been very reliable (DiCesare et al., 1997; Hummel et al., 1998; Neidhart et al., 1997; Recklies et al., 1998).

In contrast, elevated COMP levels in serum have been found to correlate with the disease course of rheumatoid arthritis and osteoarthritis (Clark et al., 1999; Forslind et al., 1992; Månsson et al., 1995; Petersson et al., 1998; Sharif et al., 1995). Serum COMP levels seem to increase within an individual as the disease becomes more severe. However, serum COMP levels do overlap somewhat between affected and the unaffected patient samples (Clark et al., 1999). Therefore, the quantitation of COMP levels could be

used to monitor disease progression and the effects of therapeutic interventions, not for diagnosis.

Observations on COMP metabolism suggests that interactions between COMP and other matrix proteins may be important for cartilage maintenance and repair. As the cartilage matrix is damaged, COMP is released into the serum. A change in serum COMP levels could be due to the fragmentation of proteins from the matrix, increased protease activity or an attempt at repair by chondrocytes to produce more COMP (Ganu et al., 1998). PSACH and MED patients develop aggressive osteoarthritis at very early ages, often requiring hip replacements before the age of 30. Currently, studies have not been published on serum COMP levels from PSACH or MED patients.

FUNCTION

Despite the data that has been reported, there is a limited understanding of how COMP is assembled, its function, and how it is involved in bone development. Although COMP has significant homology to other TSP members, most of what is known about them is based on TSP1 and TSP2. COMP shares common domains, such as the EGF-like repeats, calcium binding domains and a globular carboxyl-terminal end; however, several of the proposed functions of the trimeric TSPs are governed by the amino-terminal domains. COMP does not have these domains, and therefore, is probably not involved in such interactions (Figure 2-1C). Very little is known about the functions of TSP3 and TSP4, which COMP resembles most.

The effects of COMP mutations in PSACH and MED patients suggest that normal COMP function is essential for bone growth. Most mutations identified have been found

within the calcium-binding repeats which implies that this region is important for COMP structure and function. In affected cartilage, the ultrastructure revealed enlarged lamellar inclusion bodies in the rER. This accumulation of protein suggests that synthesis is interrupted, and in turn, prevents the construction of cartilage. A matrix that is deficient in COMP may not provide an adequate cartilage template for bone material to be deposited on (Hecht et al., 1998; Maddox et al., 1997).

Unfortunately, the patient samples available for study are limited. Many experiments are not possible with human tissue, requiring the use of model systems. In order to understand the mechanism of pseudoachondroplasia, we attempted to test the hypothesis that pentamerization of COMP is required for its normal function and may also be important for its intracellular retention. By generating transgenic mice, this project focuses on a dominant-negative mutation which is expected to prevent the assembly of complete COMP molecules. By overexpressing a truncated COMP monomer in cartilage, it should be possible to interrupt skeletal development.

The transgene (tCOMP) was made to encode a truncated COMP molecule (~300bp), containing the amino-terminal coiled-coil domain, which is required for pentamer assembly and stability. The overproduction of this domain was expected to disrupt the assembly of endogenous COMP molecules creating a dominant negative effect. The extent to which truncated COMP monomers can compete with normal COMP monomers to be assembled into pentamers depends, upon the amount of transgene expression. For instance, in transgenic mice there will be two types of monomers available for pentamer formation. If mutant and normal monomers are expressed equally, the chance of a normal pentamer to form will be 2^5 or 1 out of 32 pentamers will be

normal. So, this was expected to create pentameric COMP molecules with 0 to 5 normal monomers, depending upon the extent of transgene expression. Through analyzing the phenotypes of these transgenic mice, our goals were to determine if pentamerization is required for normal COMP function and, if so, how does the cartilage morphology change and how is bone development affected when COMP assembly is altered.

RESULTS

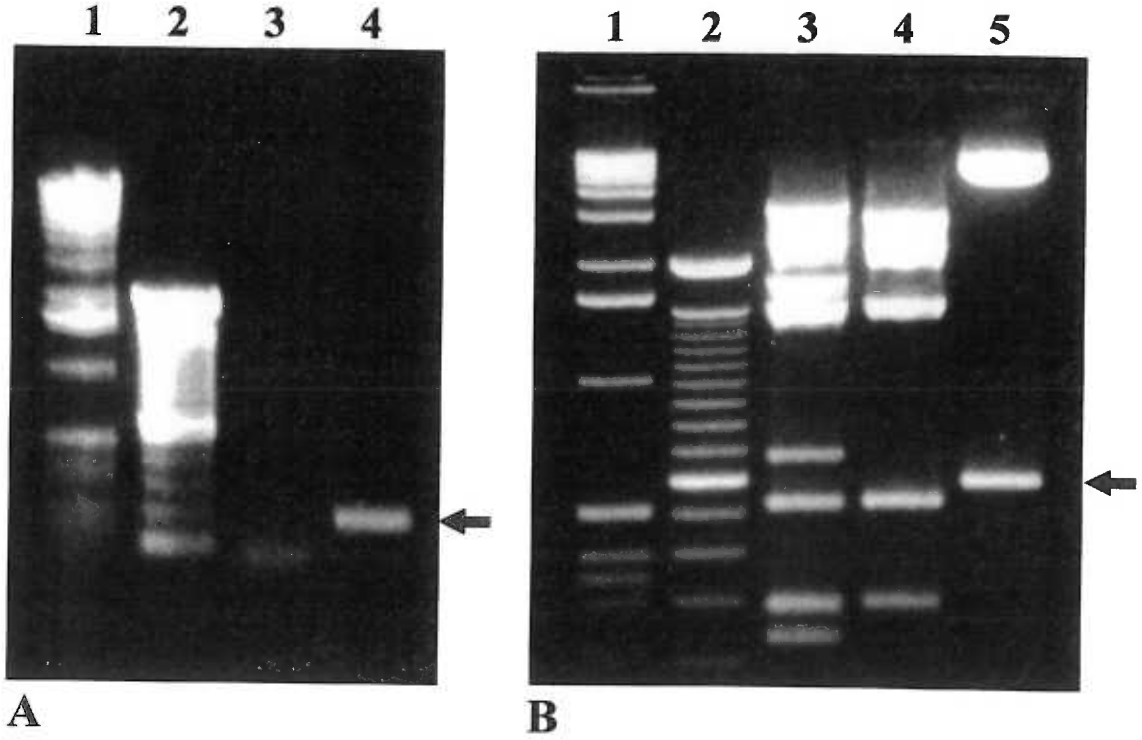
CONSTRUCTION OF THE DOMINANT NEGATIVE MUTATION

The transgene (tCOMP) encodes the amino-terminal coiled-coil domain, which is required for COMP assembly and stabilization of the pentameric form. The production of this domain is expected to disrupt the assembly of endogenous COMP molecules creating a dominant negative effect. This construct was made by using RT-PCR to amplify the coiled-coil domain from a wild-type mouse cDNA sample (Figure 2-3A). This fragment was then cloned into a pBluescript (Stratagene, Inc.) vector and sequenced (Figure 2-3B). Once the sequence was confirmed, the transgene was constructed by inserting the fragment and a polyadenylation sequence into a Col2a1 expression vector previously developed by Silvio Garofalo (Garofalo and Horton, 2000; Metsäranta et al., 1995; Zhou et al., 1995) (Figure 2-3C). This vector has been successfully used for generating mice whose transgenes are expressed specifically in cartilage (Garofalo and Horton, 2000; Garofalo et al., 1991; Maddox et al., 1997; Maddox et al., 1997; Metsäranta et al., 1995).

GENERATION OF TRANSGENIC MICE

Mice were generated by pronuclear injection of the linearized tCOMP transgene (DNX Transgenic Facility, Cranbury, New Jersey). To identify founders, tail tissues were taken from each mouse for the isolation of DNA. The transgene was detected in five males and one female by PCR (Figure 2-4B). Southern analysis, using the coiled-

Figure 2-3. Truncated-COMP construct. (A) The 300-bp PCR fragment (arrow), amplified from a wild-type mouse limb cDNA sample, is shown in lane 4. Loaded in Lanes 1 and 2 are size ladders, 1kb and 100bp, respectively. Lane 3 is an unamplified sample. (B) Digests of the final construct, *Hind III-Eco RI* (lane 3), *EcoRI-XhoI* (lane 4), *HindIII-XhoI,* 600bp fragment (arrow) (lane 5). Among the several bands in lanes 3 and 4, there is a ~300bp band in each lane which corresponds to the insert fragment. In lane 5, the ~600bp band (arrow) corresponds to the full insert fragment. Lanes 1 and 2 contain size standards, 1kb and 100bp ladders, respectively (Gibco BRL). (C) A diagram of the transgene construct.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1 2 3 4 5
A
B

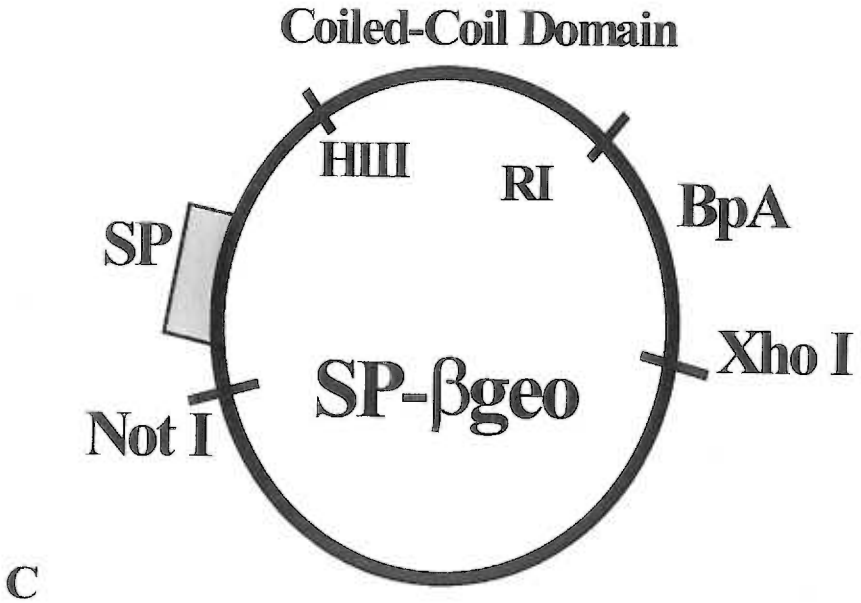
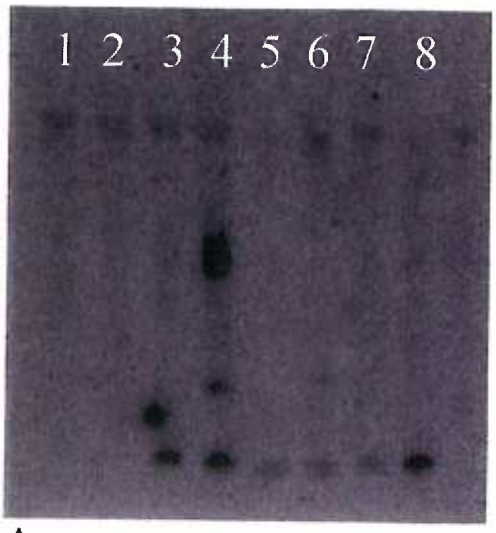

Coiled-Coil Domain
HIII
RI
BpA
SP
Xho I
SP-βgeo
Not I
C

Figure 2-4. Genotype and phenotype of transgenic mice. (A) Identification of transgenic founder mice. Genomic DNA from two wild-type mice (lanes 1-2) and from six founder mice (lanes 3-8) were digested with *Eco RI* and analyzed by Southern blot. The 600bp fragment is diagnostic of the transgene, whereas the larger fragment represents the endogenous gene. (B) PCR was also used to identify the founder mice as well as their offspring. Fragments from two wild-type mice (lanes 3-4) and from the six founder mice (lanes 5-11) are shown. The 600bp fragment represents the transgene. (C) Newborn wild-type (left) and transgenic (right) mice. (D) Cleared skeletons of wild-type (left) and transgenic (right) newborn mice. (E) Three week old wild-type (left) and transgenic (right) mice. Wild-type and transgenic mice, at newborn and three weeks of age, are very similar in size.



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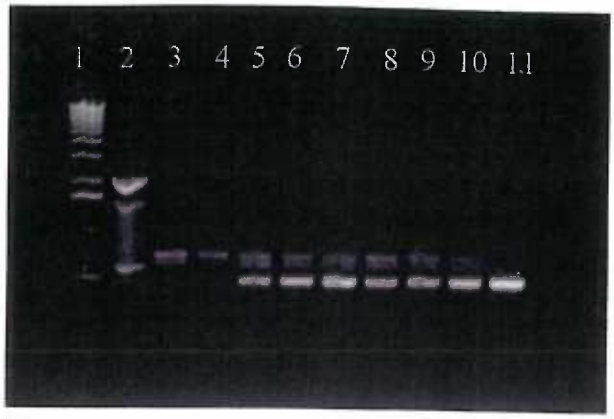


B

C

D

E

coil domain as a probe, was utilized for confirmation and to determine copy number (Figure 2-4A). All of the founder mice appeared to be normal with no adverse phenotypic effects from the transgene. The copy numbers ranged from 4 to 38. Five distinct founder mice, with 5 to 15 copies, gave rise to F_1 transgenic offspring. The offspring from the female founder, with 38 copies, were not available for study (lane 8).

ANALYSIS OF MICE WITH THE TRUNCATED-COMP TRANSGENE

The transgenic offspring from four of the breeding founders were phenotypically normal. The transgenic pups were healthy, active and fertile (Figure 2-4C, 4E). One male founder had unusually small litter sizes, one to four pups. Analyzing the embryos showed that some died in utero as early as 6 dpc, before cartilaginous tissues are present and before the transgene is expressed. Because this phenotype was only observed in one of the six founders, the lethality was attributed to insertional mutagenesis. This founder line was not included in the study.

Since there were no observable differences between wild-type and transgenic F_1 littermates, the heterozygotes were bred to yield homozygotes. This cross was expected to double the copy number and increase the level of transgene expression. However, still, there was no observable difference between the wild-type, heterozygous or homozygous transgenic mice.

Initially, transgenic mice appeared to be smaller than normal littermates. A statistical analysis of measurements from adult mice was performed to determine if there was a correlation between skeletal size and transgenic status. Wild-type and heterozygous transgenic mice, 191 total (5 months of age), of the same sex were X-rayed

and weighed. The results for weight, spine length and femur length were analyzed using the SYSTAT statistics program (version 8.0 for windows) (SPSS, 1998; Wilkinson, 1998). Measurements of offspring, from each the four founders, were comparable. To increase the total number of subjects, results from the higher copy number founders' offspring (11 and 15 copies) were combined, giving 91 mice.

The results of Pearson Chi-square analysis are shown in Table 2-1 (females) and 2-2 (males). For size differences to be statistically significant, the p-value must be below 0.05. Pearson Chi-square analysis of female mice shows a p-value of 0.13 for femur length, 0.72 for spine length and 0.27 for total weight. Analysis for male mice shows a p-value of 0.33 for femur length, 0.11 for spine length and 0.16 for weight. The p-values for both males and females, in all categories, were greater than 0.05, indicating that the transgene did not influence skeletal size. In addition, older mice (males and females at 16 months of age), wild-type and transgenic, were also studied for symptoms of arthritis. Unlike arthritis mouse models, no joint inflammation, discomfort or inactivity was observed (Fässler et al., 1994; Holmdahl et al., 1986; Holmdahl et al., 1993).

Cleared Skeletal Staining

Historically, skeletal abnormalities have been studied by staining cleared skeletons with alcian blue and alizarin red, which detect the presence of the glycosaminoglycan component of proteoglycans (cartilage) and calcified tissue (bone), respectively. After the skin and internal organs were removed from wild-type and transgenic newborn mice, their skeletons were cleared in 1% KOH and stained (Figure 2-4D). Through visual comparisons, between wild-type (left) and transgenic (right)

Table 2-1. Statistical analysis of skeletal measurements in wild-type and transgenic female mice. X-rays were taken of five month old wild-type and transgenic mice. The spine and femur lengths measured from X-rays are shown in column B and C, respectively. The mice were weighed as well, column A. Pearson Chi-square analysis shows a p-value of 0.13 for femur length, 0.72 for spine length and 0.27 for total weight. These results suggest that there is no correlation between mice having the transgene and a smaller size.

Table 2-2. Statistical analysis of skeletal measurements in wild-type and transgenic male mice. Pearson Chi-square analysis shows a p-value of 0.33 for femur length, 0.11 for spine length and 0.16 for weight. These results suggest that there is no correlation between mice having the transgene and a smaller skeletal size.

Table 1

Normal Females	WEIGHT (g)	SPINE (cm)	FEMUR (cm)
N of cases	21	21	21
Minimum	19.600	5.300	1.400
Maximum	25.000	6.200	2.000
Range	5.400	0.900	0.600
Median	23,500	5.900	1.700
Mean	22.748	5.862	1.695
Standard Dev	1.878	0.260	0.160
Variance	3.527	0.067	0.025
Transgenic Females			
	WEIGHT (g)	**SPINE (cm)**	**FEMUR (cm)**
N of cases	23	23	23
Minimum	19.500	5.400	1.500
Maximum	26.000	6.200	1.800
Range	6.500	0.800	0.300
Median	21.900	5.900	1.600
Mean	22.374	5.848	1.591
Standard Dev	1.591	0.221	0.095
Variance	2,531	0.049	0.009
Pearson Chi-square p-value	0.270	0.72	0.130

Table 2

Normal Males	WEIGHT (g)	SPINE (cm)	FEMUR (cm)
Nof cases	28	28	28
Minimum	23.400	5.800	1.500
Maximum	33.800	6.500	2.100
Range	10.400	0.700	0.600
Median	31.500	6.200	1.600
Mean	30.943	6.175	1.675
Standard Dev	2.567	0.169	0.176
Variance	6.590	0.029	0.031
Transgenic Males			
	WEIGHT (g)	**SPINE (cm)**	**FEMUR (cm)**
N of cases	19	19	19
Minimum	26.000	5.700	1.400
Maximum	34.100	6.300	1.900
Range	8.100	0.600	0.500
Median	29.200	5.900	1.600
Mean	29.179	5.968	1.621
Standard Dev	2.577	0.183	0.140
Variance	6.638	0.033	0.020
Pearson Chi-square p-value	0.16	0.11	0.33

littermates, the same bones were observed to be of similar length and degree of calcification.

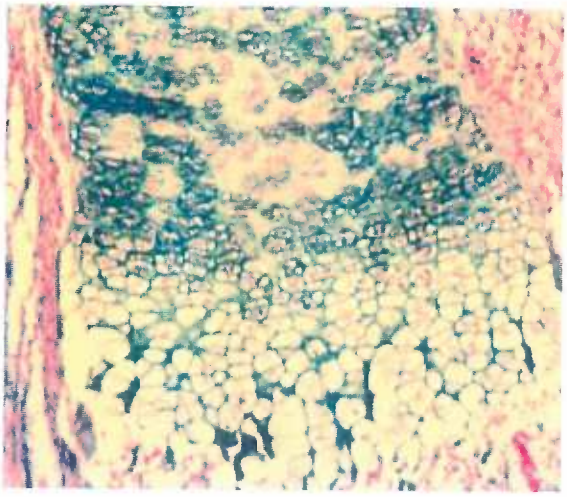
Structural and Ultrastructural Analysis of Cartilages

Using histological methods and microscopy, the developmental stages of chondrocytes can be visualized in the epiphyseal growth plate cartilage of long bones. Hind limbs were fixed in 4% paraformaldehyde, embedded in paraffin, sectioned and stained with hematoxylin, eosin and alcian blue (Figure 2-5). Transgenic (Figure 2-5B, 5D) growth plates were composed of sufficient matrix and the cellular zones were organized when compared to wild-type littermates. Hypertrophic chondrocytes were observed in a typical columnar appearance (Figure 2-5C, 5D). In addition, no morphologic or structural differences were observed in the proliferative and resting chondrocyte zones.

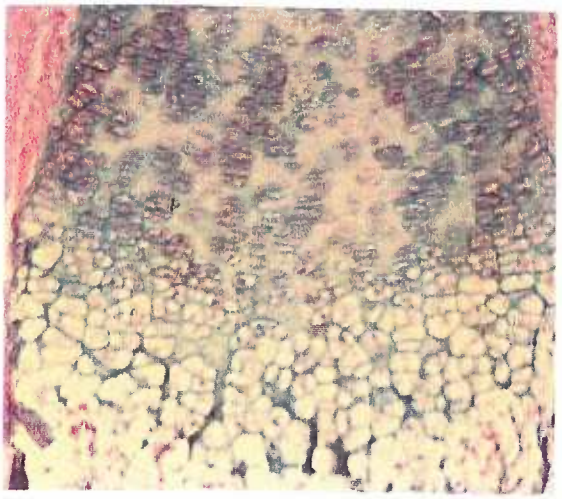
Immunostaining was carried out on paraformaldehyde fixed, paraffin embedded sections of newborn wild-type and transgenic hind limbs using a monoclonal antibody against mouse collagen type II (Figure 2-6A, 6B) and a polyclonal antibody against human COMP (Figure 2-6C, 6D). Type II collagen staining was normal, highlighting the matrix in both samples (Figure 2-6A, 6B). COMP staining was found primarily in the proliferative zone where it was mainly localized to the territorial matrix surrounding the cells (Figure 2-6C, 6D). This staining pattern has been observed in immature porcine joint and growth plate cartilage tissues (Ekman et al., 1997).

Electron microscopy was used to examine the ultrastructure of cartilage cells and matrix. Fore limbs, from wild-type and transgenic littermates were dissected

Figure 2-5. Histological analysis of the growth plate cartilage. Wild-type (A,C) and transgenic (B,D) growth plate, from newborn proximal tibia, stained with hematoxylin, eosin and alcian blue. The intensity of alcian blue staining often varies from specimen to specimen depending on loss of proteoglycan during tissue processing. The differences observed here are not considered significant. A and B are magnified 100X, C and D are magnified 400X.

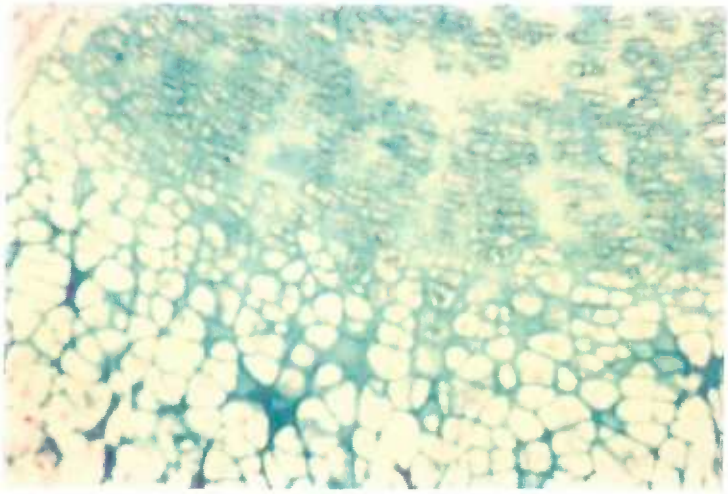


A



B

C



D

Figure 2-6. Immunohistochemistry of growth plate cartilage. Using an antibody against type II collagen, newborn wild-type (A) and transgenic (B) samples were analyzed (magnification 20X). Wild-type (C) and transgenic (D) proximal tibia cartilages are stained with antibodies against COMP (magnification 40X). The distribution of type II collagen and COMP in the matrix is normal in transgenic samples. In transgenic tissues, intracellular COMP staining was comparable to wild-type tissues.

A

B

C

D

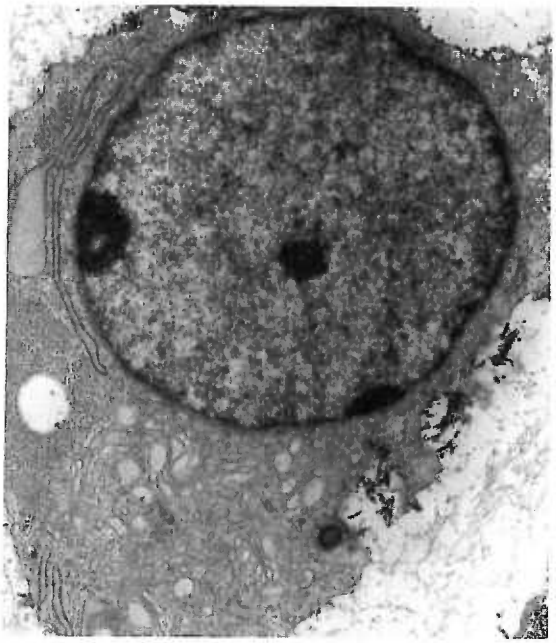
immediately after birth, fixed, dehydrated in ethanol and embedded in Spurr's epoxy resin. Ultrathin sections were examined. Transgenic samples (Figure 2-7B, 7D, 7F) showed the normal amount and size of fibrils, when compared to wild-type tissues (Figure 2-7A, 7C, 7E). Both displayed prominent rER compartments typical of biosynthetically active chondrocytes.

TRANSGENIC COMP PRODUCTION AND SECRETION

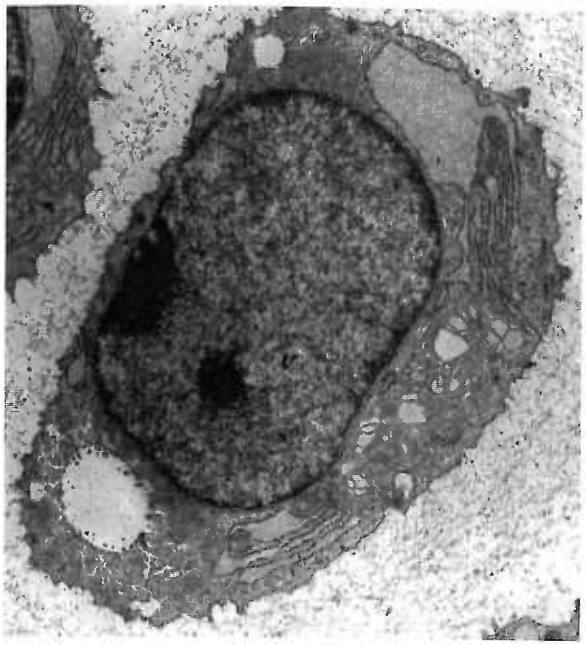
Western analysis was performed to determine the presence of normal pentameric COMP, in wild-type mice, and the incomplete COMP molecules in the transgenic mice. Because the endogenous full length monomers are predicted to assemble with the transgenic truncated monomers, a ladder of proteins, with progressively smaller molecular weights was expected to be present in transgenic samples. Rib chondrocytes were obtained from newborn wild-type and transgenic mice. After pronase and collagenase digestion, the chondrocytes were cultured overnight in DMEM with fetal calf serum. On the following day, the cells were washed and then media with ascorbic acid and TGF-β1 was replaced. After culturing overnight, cell and media fractions were taken. Protease inhibitors (Sigma cocktail #2 and DFP) were added. A polyclonal antibody to human COMP was used for Western analysis (Figure 2-8). When compared to the purified human COMP control (lane 1), pentameric COMP (arrow) was present in all samples. In addition, smaller bands with faster migration were detected in both wild type and transgenic samples. There were no differences observed between wild-type and transgenic protein samples. Rat COMP coiled-coil domain antibodies were also utilized;

however, after attempts to optimize recognition, these reagents did not detect the transgenic or endogenous COMP coiled-coil domain (R Perris, Genoa, Italy).

Figure 2-7. Electron microscopy of cells and matrix from cartilage of newborn mice. Newborn wild-type (A) and transgenic (B) chondrocytes from the resting zone of the growth plate (25,000X). Wild-type (C) and transgenic (D) matrix surrounding chondrocytes in the proliferative zone (25,000X). Wild-type (E) and transgenic (F) chondrocyte rough endoplasmic reticulum (40,000X). There is not a clear difference ultrastructurally in the transgenic samples when compared to wild-type cartilage.

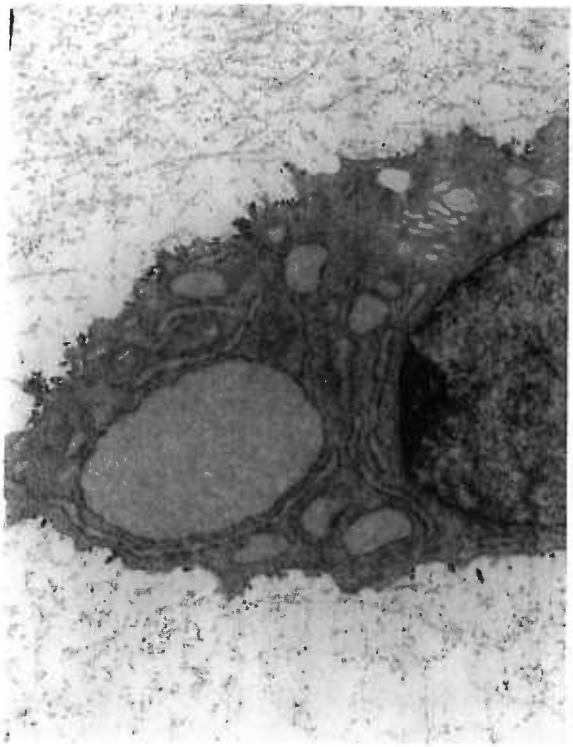


A

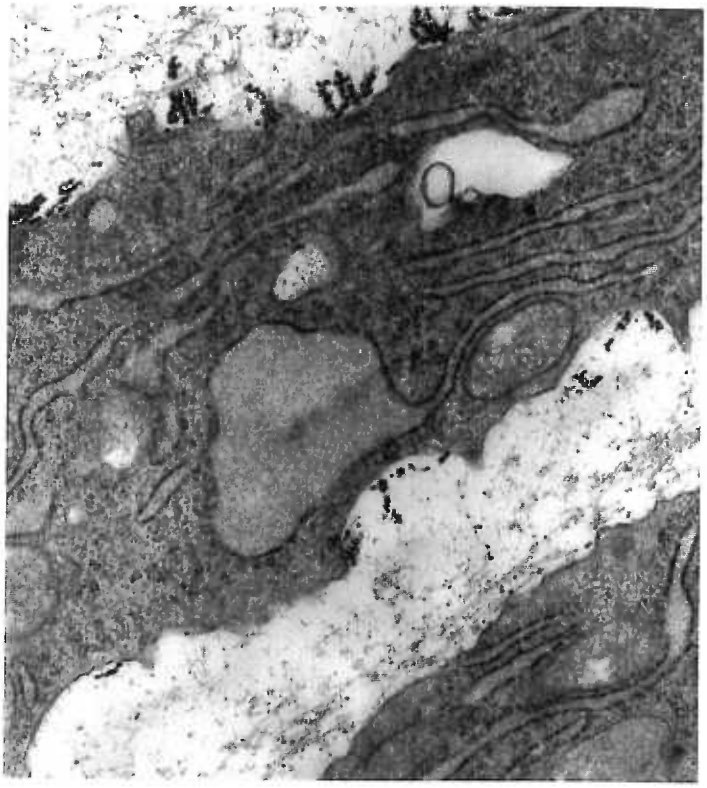


B

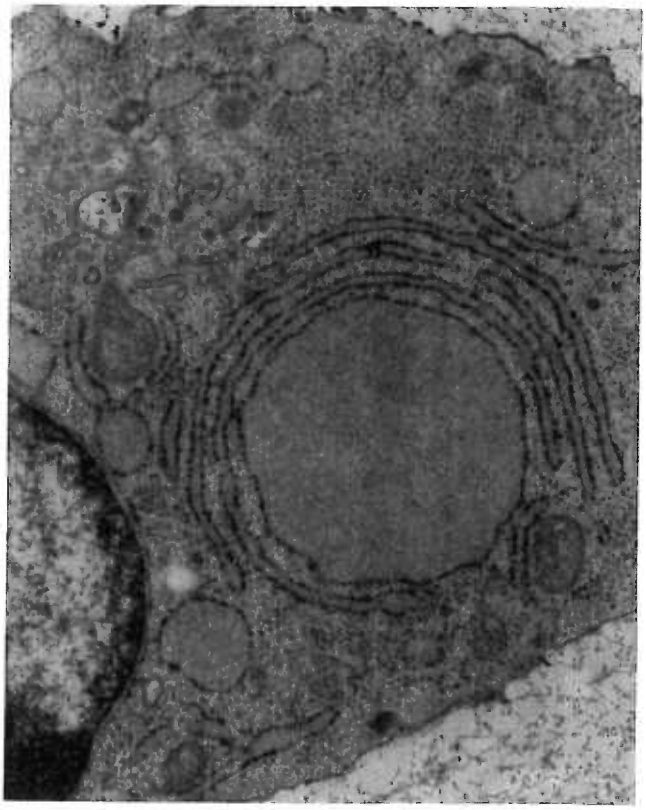
C



D

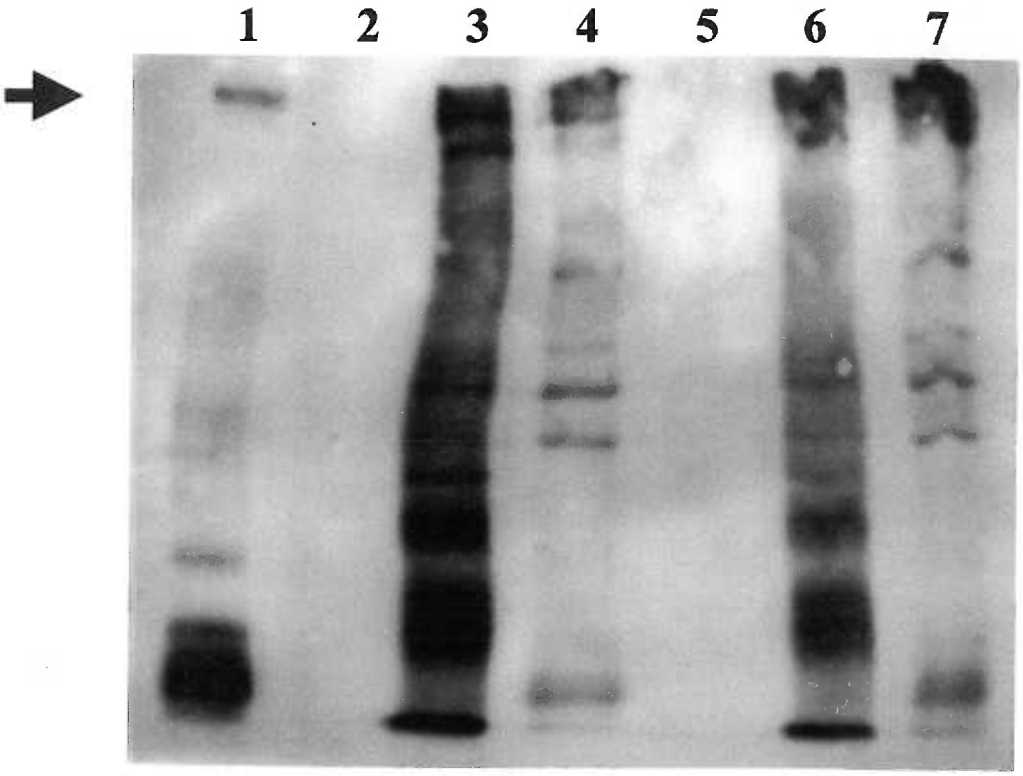


E



F

Figure 2-8. Analysis of COMP and transgenic product. Western analysis of wild-type and transgenic rib chondrocytes are shown using a polyclonal antibody to human COMP. Pentameric COMP (arrow), corresponding to the purified human control (lane 1), was present in all fractions. Additional bands are present in experimental fractions, wild-type cell (lane 3), wild-type media (lane 4), transgenic cell (lane 6) and transgenic media (lane 7). No samples were loaded in lanes 2 and 5. The banding patterns are the same in wild-type and transgenic samples. Non-reduced samples were run on 4.5% acrylamide gels.


1
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7

DISCUSSION

Chondrodysplasias are inherited disorders of cartilage that disrupt the growth and function of the skeleton. Through identifying human mutations which disrupt skeletal development, a basic understanding of the factors which govern this process is obtained. However, determining the mechanism through which a mutation causes disease requires further experiments that are difficult to perform with available patient tissue. To overcome this obstacle, the production of transgenic mice has become a powerful tool. With a mouse model available for study, biochemical, genetic and developmental questions can be answered without the need for human tissue. Through studying the normal and abnormal biology of skeletal growth in mice, the function of molecules essential for developmental mechanisms, as well as the pathogenesis of human skeletal disorders, may be explored.

Due to mutations in COMP causing PSACH and MED, COMP is thought to have an important role in bone development. Its expression in developing cartilage, as well as in adult articular cartilage, suggests that COMP is needed not only for skeletal growth and development, but also to maintain bone structure throughout the life of the organism. In this study, the truncated coiled-coil domain was over expressed with the intent of disturbing normal COMP assembly. One would expect that a dominant negative mutation that alters COMP assembly would have a profound effect on chondrogenesis and, perhaps, cartilage maintenance. It was, therefore, surprising that the transgenic mice with the truncated COMP transgene exhibited no observable abnormalities.

These mice were normal in appearance and reproduction. When compared to wild-type littermates, transgenic mice were of normal size through adult stages. In

addition, cleared skeletons of normal and transgenic newborn littermates did not reveal any differences in bone length or in calcification rates. Furthermore, growth plate morphology and COMP antibody staining were also within normal expectations. The cells, matrix and rER of transgenic samples were normal when viewed by electron microscopy.

Biochemical analysis revealed that the transgenic mice produced no truncated COMP molecules, although the transgene was incorporated into the host genome. Endogenous pentameric COMP was the primary product observed by western blotting cartilage proteins from transgenic samples. Degradation products revealed similar banding patterns when compared to normal protein samples. This indicates that pentameric COMP assembly was not disrupted or otherwise defective in the transgenic mice. There are at least three possible explanations for this result: (1) the RNA message was not stable or it was expressed at very low levels, (2) the truncated monomers were proteolytically degraded before assembling with endogenous COMP monomers or (3) truncated COMP monomers did not assemble with endogenous monomers.

First, it is possible that the RNA transcript from the transgene is degraded before translation can occur. However, similar sequences from TSP1 and cartilage matrix protein (CMP) encoding coiled-coil domains alone have successfully been expressed in other cell lines. In each case, the construct produced a truncated protein that assembled with other truncated and normal endogenous monomers into trimers (Chen et al., 1995; Sottile et al., 1991). In addition, there is the possibility that the transgene is not expressed highly enough. This is unlikely, as well, because the construct used to generate the mice was governed by the mouse collagen type II promoter, which is highly expressed in

cartilage (Metsäranta et al., 1995). However, there could be other factors, unrelated to the construct, that cause decreased expression, such as the insertion of the transgene into a region near a silencer or heterochromatic region.

Secondly, it is possible that the truncated monomers are translated but degrade before they assemble into complete pentameric molecules. Very little is known about COMP assembly and the molecules involved in its processing. However, coiled-coil domains made up of closely related sequences are found in many different molecules and once formed, result in extremely stable structures. In a related protein, TSP1, it is known that it's monomers are assembled into trimeric molecules in the rER before the associated monomers are completely translated (Prabakaran et al., 1996). This leaves little time for protein degradation before assembly is complete. In addition, truncated amino-terminal monomers containing the coiled-coil domain have been shown to form heterotrimers with endogenous TSP1 (Sottile et al., 1991). It was, therefore, expected that truncated COMP containing the amino terminal coiled-coil domain would also combine with endogenous COMP chains to form a heteropentamer.

Thirdly, it is possible that the truncated monomers prefer to form pentameric structures with itself, leaving the endogenous monomers undisturbed. With the COMP antibodies available, a transgenic product, in pentameric or monomeric form, was not detected. COMP antibodies can not currently be obtained commercially; therefore, I have had to rely on other resources. Polyclonal and monoclonal antibodies to human COMP were used for western blot analysis (Lynn Sakai, Portland, OR) (Maddox et al., 1997). The polyclonal antibody detected mouse COMP molecules under nonreducing conditions, but not with reduced material. The monoclonal antibody did not detect

mouse COMP in any, nonreduced or reduced, protein samples. Antibodies made specifically against the rat COMP coiled-coil domain were also used in western blot analysis (R Perris, Genoa, Italy). After attempts to optimize antibody recognition, these reagents did not detect the transgenic or endogenous COMP coiled-coil domain. The antibodies to rat COMP have only previously been used for ELISA experiments, not for western blot analysis.

Since I was unable to demonstrate that the transgene product was made or incorporated into COMP pentamers *in vivo*, a similar experiment was done *in vitro*. The tCOMP fragment, used in the mouse construct, was cloned into the pCDNA3 vector (Invitrogen, Inc.). This vector, governed by a CMV promoter, was transfected into COS cells. The protein extracts were collected and western blot analysis was done to verify that a transgenic product was present. Because COS cells do not produce COMP endogenously, only truncated monomers would be available for assembly. In spite of positive transfection controls in which other vectors were transfected into COS cells in parallel, the truncated-COMP protein, in monomeric or pentameric form, could not be detected with any of the available antibodies (data not shown).

It is very likely that the reagents available can not identify the transgenic encoded truncated-COMP protein. Prior to making the mouse construct, there was much discussion about the efficacy of inserting a recognition tag (myc, his or FLAG) adjacent to the coiled-coil domain. This would have allowed use of commercially available antibodies to recognize the transgenic product. On the other hand, the addition of a peptide might have inhibited COMP assembly. At the time, the available human COMP

antibodies were known to identify COMP in mouse tissue and to work with western blot analysis.

SUMMARY AND CONCLUSIONS

COLLAGEN TYPE II R789C MUTATION

A patient with SED congenita with moderately severe chondrodysplasia was reported to have a *COL2A1* mutation, R789C. One of the experimental goals was to model SED congenita in transgenic mice and to study their skeletal development. Transgenic mice had severe dwarfism with a delay in skeletal development. They died at birth due to respiratory complications. The affected embryos had short limbs and trunk, a distended abdomen, a small thorax, a short snout, a protruding tongue and cleft palate. Although the mutant mice had skeletal abnormalities, their chondrodysplastic phenotype was unexpectedly much more extreme than the human condition.

Histologically, the most striking difference between the transgenic and wild type littermates was the disorganization of the growth plate. There was also a decreased number of chondrocytes, particularly in the proliferative zone. Immunofluorescence showed reduced staining when using antibodies that recognize type II collagen and aggrecan. Thus, collagen type X staining was considered normal. Furthermore, electron microscopy revealed that transgenic cartilage had a marked decrease of collagen fibrils in the matrix. This severe phenotype closely resembles that of the *Col2a1* null mouse (Li et al., 1995).

A second goal was to use this mouse model as a target for antisense therapy. When constructing the transgene, a silent mutation was made for the insertion of a hammerhead ribozyme cleavage site. Ribozymes and test targets were generated for *in vitro* experiments to determine ribozyme specificity. Even though the ribozyme could

discern the mutant target from the wild type target, it was not capable of cleaving all transcripts presented. Due to the unexpected severity of the transgenic phenotype, further experiments to optimize ribozyme efficiency were discontinued.

The final goal was to use this model system to understand the mechanism of a Y-position mutation in the collagen triple helix. Using molecular modeling techniques, the triple helical region surrounding the mutation was reconstructed. In agreement with similar studies, the model with the mutant cysteine residue suggested that helix assembly would not be adversely affected by this mutation (Fertala et al., 1997). In transfected cells, type II procollagen molecules were not found in the media fractions; however, reduced amounts were present in the cell/pericellular matrix fraction. Preliminary labeling experiments has shown that the mutant α1(II) chain is present in the cell/pericellular matrix fraction. Further experiments determined that procollagen molecules, containing the mutant chain, were retained inside the cells. Using cells transfected with the construct, we found no evidence of intramolecular disulfide-linked α1(II) dimers in western analysis or labeling experiments.

In conclusion, our results suggest that the R789C mutation does not interfere with triple helix assembly, but does inhibit cartilage fibril formation. The greatly decreased number of collagen fibrils is probably explained by the failure of molecules containing mutant α chains to be secreted or once secreted they are destroyed by proteolysis. There are three possible explanations why these procollagen molecules may not be available for fibrillogenesis. First, molecules containing mutant chains may not be secreted from the cell, which reduces the number of molecules available to contribute to the fibrils. Secondly, procollagen molecules composed of mutant chains may be secreted and

degraded before fibril assembly occurs. And thirdly, as previously suggested, the molecules containing the mutant chains may decrease the lag time in fibril assembly or cause instability within the fibril. Further analysis is needed to determine why fibrillogenesis is interrupted.

DOMINANT NEGATIVE COMP MUTATION

The present study was done to determine how bone development is affected when COMP function is disrupted. The intention was to overexpress the assembly domain, thus, disrupting the assembly of normal COMP molecules. After a thorough evaluation, the transgenic mice had no observable phenotype. When compared to normal littermates, they had similar skeletal features and growth plate morphology with no evidence of premature cartilage degeneration. Transgenic growth plates had normal COMP staining by immunofluorescence, while mutant cartilage matrix and chondrocyte cell morphology was unremarkable when viewed by electron microscopy.

Although the transgene status was established, the presence of truncated COMP monomers was not confirmed. By western analysis, endogenous pentameric COMP was present in wild type and transgenic samples. This result suggested that endogenous COMP assembly was not disturbed in cultured mutant mouse chondrocytes. Additional lower molecular weight bands were also present. However, because the truncated product could not be confirmed and these lower weight bands were present in all samples, they are probably degradation products. Due to species differences, it is unfortunate that the available antibodies could not detect the truncated COMP monomer.

There are three possible explanations as to why endogenous COMP assembly is not disturbed. First, the transgenic RNA message could be unstable or expressed at a very low level. A second possibility is that the truncated monomers are proteolytically degraded before assembling with endogenous COMP monomers. Thirdly, the truncated monomers may only form pentamers with itself and not incorporated with endogenous monomers.

Very little is understood about normal COMP assembly and function. Most of the information available, is based on the resulting phenotypes caused by COMP mutations in skeletal disorders. However, extrapolating normal COMP function based on these disorders is difficult, due to the other proteins involved and the abnormal cellular phenotype. It is interesting to note that PSACH chondrocytes are typically surrounded by a very sparse, but normal appearing matrix with collagen fibrils and proteoglycan aggregates (Hecht et al., 1998). This could suggest that in the absence of COMP normal matrix formation may not be disturbed or other proteins exist that have similar roles. Many questions about normal COMP function still remain and further studies will need to be done to determine COMP function.

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APPENDIX 1

MOLECULAR MODELING

Methods

Molecular modeling was performed on a Silicon Graphics Indigo 2® workstation using software programs from MSI of San Diego. The region around the R789C mutation was modeled using structure RS ((Ac-(Gly-Pro-Pro)$_4$-NHMe)$_3$) as a template for the backbone conformation [Nemethy, 1992 #289]. The replacement of the amino acids in this structure for residues 784 to 795 was accomplished using the *Biopolymer*® program. Side chain conformations were optimized by manually selecting the lowest energy conformer for each amino acid (except the prolines and glycines, which were not substituted) within the *Biopolymer*® program. Energy minimizaton calculations were done with the *Discover*® program, using the CVFF force field. The backbone was tethered in place throughout the calculations with a force constant of 100 kcal/Å^2. A distant-dependent dielectric constant of 1.0 was used throughout the calculations and the side chains of all amino acids were uncharged, in order to approximate the effects of solvent shielding [Vitagliano, 1993 #290]. The protocol for the minimization was as follows: the method of steepest descents was used initially until a maximum derivative of less than 10 kcal/Å was reached, with a charges term included. Next, the method of conjugate gradients was used for 500 iterations until a maximum derivative of less than 1.0 kcal/Å, with charges and cross term energies included. Finally, the va09a method, which is a quasi-Newton-Raphson method, was used for 500 iterations until a maximum

derivative of less than 0.01 kcal/Å was achieved, with charges, cross terms, and a morse bond potential included.

Results

The model of the region around the mutation is shown in Figure 8 using the *Insight II*® molecular modeling system. Overall, the conformations of the side chains were the same for both the wild-type and mutant molecules. Since the R789C mutation may affect the local stability around this residue, we measured the non-bonded residue-residue interaction energies with the *Discover*® program. The energies, which include both van der Waals and coulombic interaction energies, were measured between R789 (and R789C) and all other residues in the molecule. The energies between R789 and those residues immediately surrounding this are shown in Table 1, as well as the energies for the mutant R789C. Of significance are the energies between R789 and E791 (Glu A9, B9), which form close contacts in the calculated structure. The total energies for this pair are approximately -5.6 kcal/mol lower than the mutant pair, and are mainly due to favorable coulombic interactions. The total van der Waals interaction energies are negative for all pairs. However, the wild-type Arg789->Phe794 (ArgC7->PheA12) interaction energy is -2.37 kcal/mol lower than the mutant. Thus, based upon these calculations, the R789C mutation would be expected to destabilize the triple-helix, mainly by disrupting favorable coulombic interactions as well as to a lesser extent favorable van der Waals interactions.

Appendix A.1. Molecular model of the triple-helical region around Arg 789. The wild-type molecule (green) is shown overlayed on the mutant molecule (magenta). The cysteines are shown in yellow. A7 and B7 refer to Arg/Cys789 in the A and B chains, respectively.

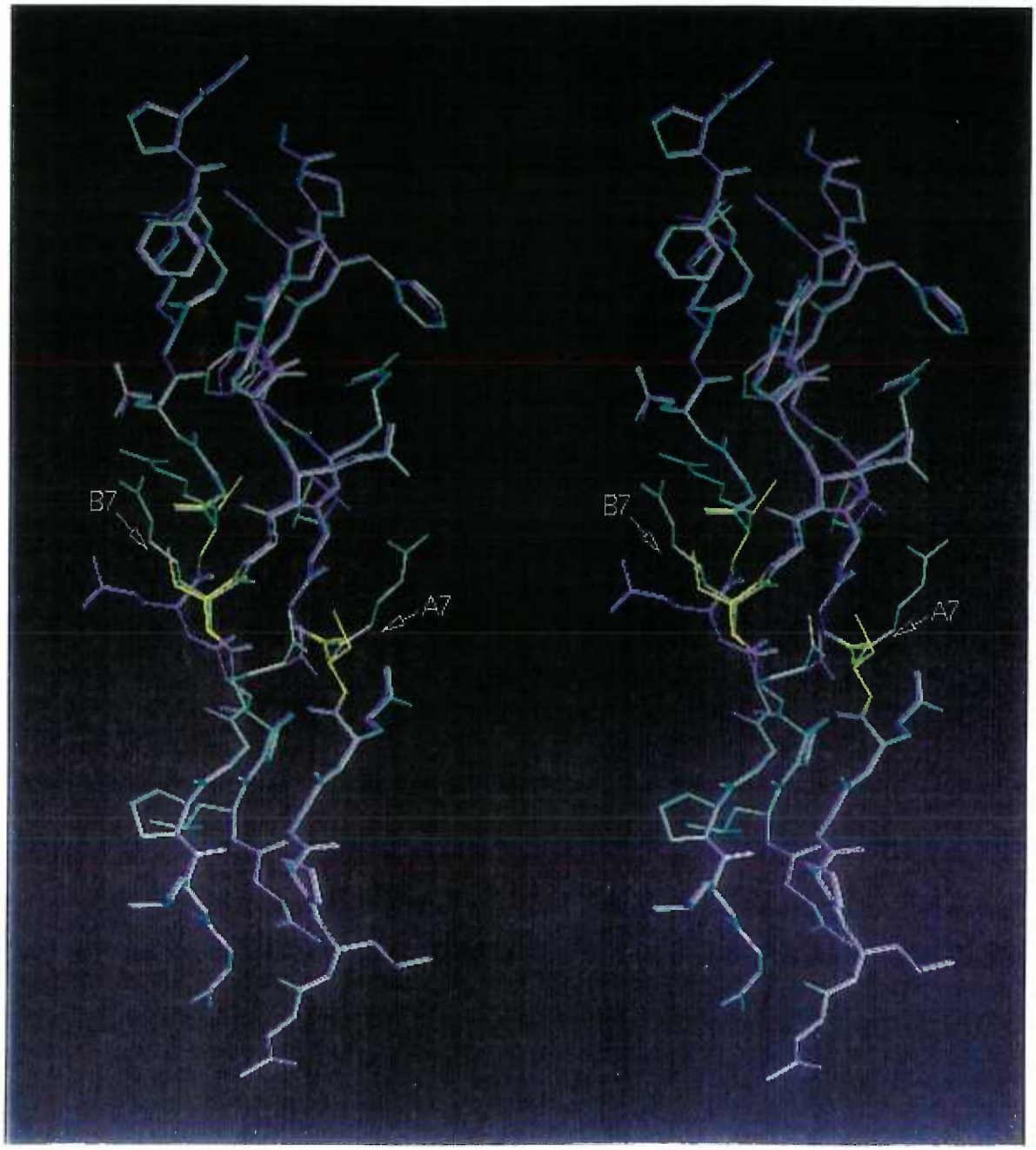
B7
A7
B7
A7

Table A.1 Bond energies between R789 and its surrounding residues. The energies between R789 and E791 are lower in the mutant pair compared to wild type, due to favorable coulombic interactions. The van der Waals interaction energy is higher in the mutant when considering the R789 and F794 calculations. This suggests that R789C would destabilize the collagen triple-helix.

Non-bonded Interaction Energies (kcal/mol)					
Res.->Res.*	Total Energy	van der Waals			Coulombic
		Repulsion	*Dispersion*	*Total*	
ArgA7->GluB9	-5.76823	3.6974	5.6452	-1.94781	-3.82042
ArgA7->GlnB6	-4.35638	3.332	6.561	-3.22905	-1.12733
ArgB7->GluC9	-5.80533	3.8494	5.7854	-1.93603	-3.86931
ArgB7->GlnC6	-4.52246	3.1909	6.4412	-3.25033	-1.27213
ArgC7->GluA9	-4.66104	2.8024	5.9985	-3.19618	-1.46846
ArgC7->PheA12	-3.40685	2.3858	5.3245	-2.93867	-0.46818
CysA7->GluB9	-0.17749	0.0061	0.2002	-0.19415	0.01666
CysA7->GlnB6	-4.14471	2.7798	5.6928	-2.91296	-1.23175
CysB7->GluC9	-0.17355	0.0055	0.195	-0.18946	0.01591
CysB7->GlnC6	-4.16802	2.7387	5.5844	-2.84569	-1.32233
CysC7->GluA9	-4.31934	2.2512	2.2512	-2.57171	-1.74763
CysC7->PheA12	-0.57155	0.032	0.5991	-0.56709	-0.00446

** The single chains (A, B and C) begin with the acetyl group as A1, B1, and C1.*

APPENDIX 2

MOUSE COMP COILED-COIL DOMAIN SEQUENCE

TAT.CGA.**TAA.GCT.TG**A.TGC.AGC.TCC.GCC.GCC.**ATG**.GGC.CCC.ACT.GCC.

Hind III *Start*

TGC.GTT.CTA.GTG.CTC.GCC.CTG.GCT.ATC.CTG.CGG.GCG.ACA.GGC.CAG.

GGC.CAG.ATC.CCG.CTG.GGT.GGA.GAC.CTG.GCC.CCA.CAG.ATG.CTG.CGA.

GAA.CTT.CAG.GAG.ACT.AAT.GCG.GCG.CTG.CAA.GAC.GTG.AGA.GAG.CTG.

TTG.CGA.CAG.CAG.GTC.AAG.GAG.ATC.ACC.TTC.CTG.AAG.AAT.ACG.GTG.

ATG.GAA.**TGT**.GAT.GCT.**TGC**.GGA.ATG.CAG.CCC.GTA.CGC.ACC.CCC.GGT.

Cys *Cys*

CTG.AGC.GTG.**TGA**.CCA.TGG.CGC.ATC.**GAA.TTC**.CTG

Stop *Eco RI*

Appendix 2. Sequence analysis of the mouse coiled-coil domain. The sequence shown was cloned into pBluescript (Stratagene, Inc.) using *HindIII* (base 53) and *EcoRI* sites (base 340). The coiled-coil sequence began at the ATG codon (base 76).